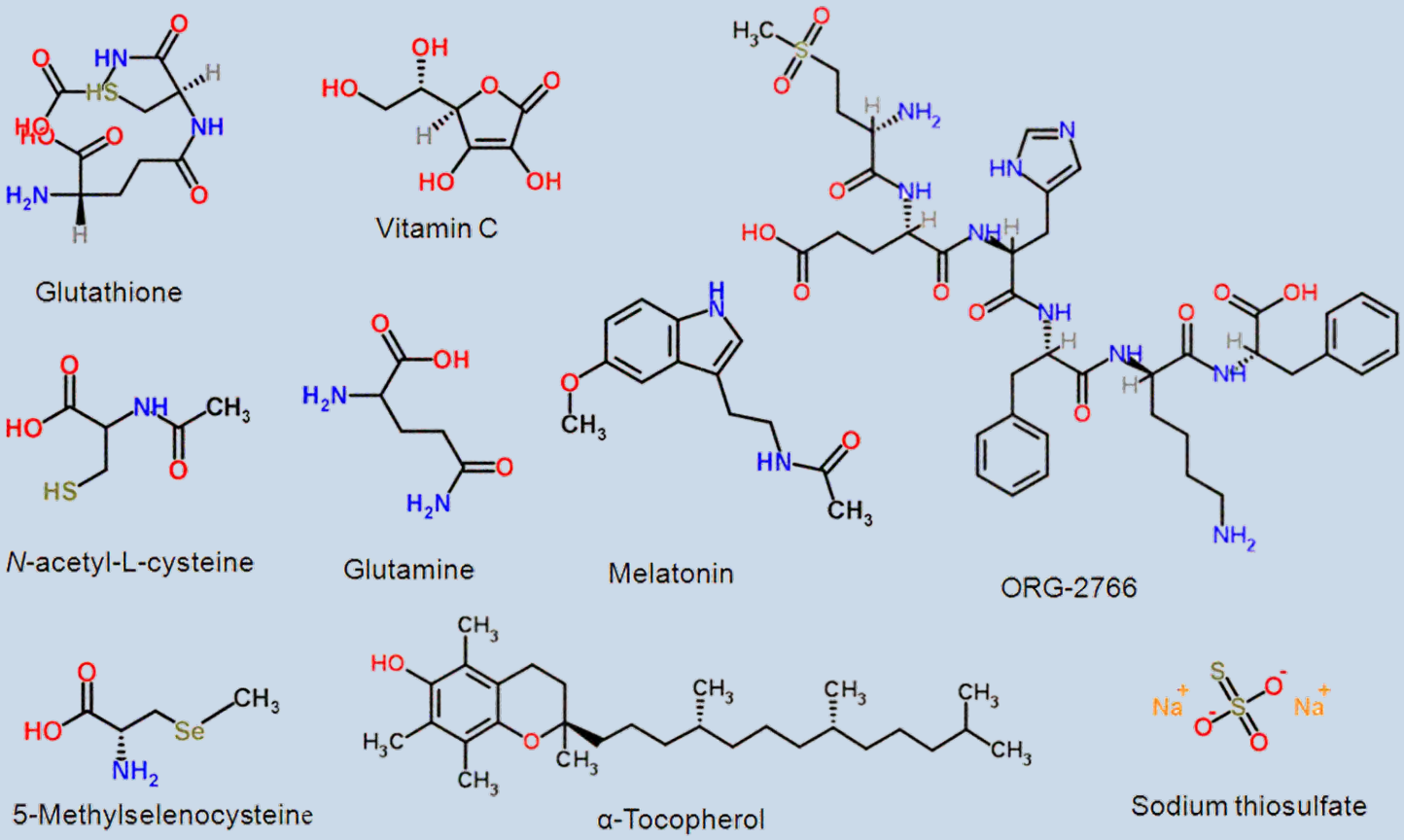
SVKM'S NMIMS Deemed to be UNIVERSITY

SUNANDAN DIVATIA SCHOOL OF SCIENCE

Biomedical Research Journal

OCTOBER 2016 | VOLUME 3 | ISSUE 2 pISSN: 2349-3666; eISSN: 2349-3674



Chemical structure of some clinically relevant chemoprotective agents

INDEXED WITH: EBSCO*host*, Index Copernicus, Google Scholar, HINARI, CiteFactor, DRJI, Advanced Sciences Index

SVKM'S NMIMS Deemed to be UNIVERSITY

SUNANDAN DIVATIA SCHOOL OF SCIENCE

Biomedical Research Journal

OCTOBER 2016 | VOLUME 3 | ISSUE 2

EDITORIAL OFFICE

Sunandan Divatia School of Science,
NMIMS (Deemed-to-be University)
Bhaidas Sabhagriha Building, Bhaktivedanta Swami Marg,
Vile Parle (W), Mumbai 400056, India.
Email: brj.sos@nmims.edu

EDITORIAL ASSISTANT

Brijesh S. (Mumbai, India)

Biomedical Research Journal

General Information

Aims and Scope

"Biomedical Research Journal (BRJ)" is a premier peer reviewed open access journal, published by Sunandan Divatia School of Science, NMIMS (Deemed-to-be) University, for promoting the advancement of ideas in the interdisciplinary realms of Medicine, Science and Technology. The goal is to share new discoveries and translational knowledge with scientists, academicians, clinicians and students in the field of Biomedical and Biology/Chemistry/ Biotechnology/Stem Cell Biology/Cancer Biology in the realm of basic and applied aspects in the different areas.

BRJ aims at creating a platform to help advance the domains and frontiers of inter- and multi-disciplinary research across the various areas of sciences and recent advances in cross pollination across biology, chemistry, and medicine. Integrative science is the present and future of science, and the journal proposes to highlight and emphasize contemporary technology towards understanding various aspects of the sciences.

The initial focus areas of BRJ include review articles and original research papers in cancer biology, stem cell biology, nano-technology and phytochemistry.

A rigorous peer review process is implemented to judge the effectiveness, legitimacy and reliability of the research content. The papers will be published online as well as provide hard copy of the Journal issues to the authors of the papers on request.

Information for Subscribers

BRJ is planned as a six monthly publication with two issues published in the first year. Currently, there are no subscription charges for the journal and can be accessed online. For submission instructions, subscription and additional information please visit: http://science.nmims.edu

Disclaimer

The views and opinions expressed in the articles published in the journal are the sole responsibility of the authors. The Publisher, NMIMS School of Science and the Editors cannot be held responsible for errors or any consequences from the use of information contained in this journal.

Contents

October 2016, Volume 3, Issue 2

BRJ
Biomedical Research Journal

BRJ
Biomedical Research Journal

Editorial

Dhananjaya Saranath and Aparna Khanna

Sunandan Divatia School of Science, NMIMS (Deemed-to-be) University, Vile Parle (W), Mumbai - 400056, India.

Come October and all in the Biomedical field await the announcement of the Nobel laureates in our fields. The 2016 Nobel Prize in Physiology or Medicine was awarded to Professor Yoshinori Ohsumi, for his discoveries of mechanisms underlying autophagy. The Nobel Prizes are announced for the most important discoveries for the benefit of mankind, at Karolinska Institutet, Stockholm. Dr. Ohsumi, Ph. D., from University of Tokyo, Batch of 1974, did a three year post-doctoral at Rockefeller University, New York, USA, and later established his research team at the Tokyo Institute of Technology. Dr. Ohsumi discovered and elucidated mechanisms underlying autophagy, a fundamental process for degrading and recycling cellular components. In the 1990's, Yoshinori Ohsumi, envisaged a series of innovative experiments using baker's yeast to identify genes essential for autophagy, and unravelled the mechanisms for autophagy initially in yeast and confirmed the process in mammalian cells.

Ohsumi's discoveries led to a paradigm shift with respect to the concept of recycling the content of mammalian cells. His discoveries revealed the fundamental path to understand the role of autophagy in several physiological processes, particularly in response to stress due to starvation, response to infection and other stresses. Mutations in genes associated with autophagy often leads to diseases including infections, neurological diseases and cancer. The Nobel laureate built his dogma on degradation as a critical function in living cells, with the lysosome organelle containing enzymes for digestion of cellular contents for degradation of cellular constituents. The autophagosome vesicles, engulfing cellular contents such as damaged proteins and organelles, fusing the contents/organelle with the lysosome, and degradation of the contents into smaller constituents, providing the cell with nutrients and

building blocks for renewal.

Yoshinori Ohsumi focused on protein degradation in yeast mutants in a vacuole similar to lysosome in mammalian cells, and identified 15 critical genes comprising a cascade of proteins in complex cellular pathways in autophagy. He demonstrated that the proteins regulated distinct stages of autophagosome initiation and formation. Autophagy provides fuel for energy and building blocks for renewal of cellular components during stress, and can eliminate intracellular bacteria and viruses. Autophagy contributes to embryo development and cell differentiation. Besides, autophagy eliminates damaged proteins and organelles, and provides a critical balance for the errors, wear and tear in the ageing process. Deregulation in autophagy has been associated with Parkinson's disease, type 2 diabetes, genetic diseases, age related problems in the elderly, and cancer. Dr. Ohsumi thus provided target molecules to develop drugs to target autophagy in various diseases, through his extensive research.

Another current issue in India is cervical cancer in Indian women, and hence we would like to briefly summarize the current International meeting on 'Cervical Cancer Prevention & Control in India and Beyond – A comprehensive Approach Towards Elimination', held on 16-18th October 2015, New Delhi, organised by Global Health Strategies with several international/national partners including American Cancer Society, and WOMEN DELIVER. The issue is so intense that several Non-Government Organisations working to alleviate Cervical Cancer were felicitated by the organisers 'In appreciation of their Inspiring and Enduring Commitment to Fight Against Cervical Cancer' by Hon'ble Minister of State, Ministry of Health and Family Welfare, Government of India.

The central theme of the meeting, repeatedly reinforced at the inaugural session by Dr. N. K. Ganguly, Former Director, ICMR, New Delhi, Chris Elstoft, Deputy High Commissioner, Australian High Commission, New Delhi, Dr. C. N. Purandare, President, International Federation of Gynecology and Obstetrics (FIGO), Dr. Soumay Swaminathan, Secretary, Department of Health Research, Ministry of Health and family Welfare, Government of India and Director General – ICMR was: 'Cervical Cancer is Preventable, and it is imperative to change the course of the

disease and 'Women Need Not Die of the Disease. Preventing cervical cancer is the right thing to do, the only thing to do'. The main features to be considered in order to achieve the goal needs to focus on 'Cervical Cancer Screening in Women and Uptake of Human Papilloma Virus (HPV) Vaccine in Girls'. The statistics of Cervical Cancer in India are appalling with an estimated 123,000 new cases diagnosed annually, and 67,000 deaths due to the disease, contributing 25% of the global cervical cancer incidence and death by a single country – India. We need to be aware that every eight minute an Indian woman is dying of cervical cancer in India. HPV vaccines with proven 70% prevention of cervical cancer is available and accessible to 5% women in rural India, the most vulnerable women.

The mandate and consensus with the cumulative expertise and experience of the delegates was – 'HPV vaccine should be given to girls in the age group of 10–12 years, with emphasis on School Based Campaigns'. The challenges with the health officials, doctors and various groups for implementation of screening strategies and HPV vaccination will be – Public Education, Understanding and Practice, Acceptance, Coverage and Financial/Manpower resource. An investment in 'Health Care for Women' needs to follow the government efforts in 'Maternal and Child Care' campaign with a comprehensive approach with reduction in maternal/child mortality to 50% of the figures to 44,000 deaths. A comprehensive approach will make a difference in reducing cervical cancer incidence and deaths. Ms. Barkha Dutt, Consulting Editor, NDTV, moderating the session 'Elimination of Cervical Cancer in India: A Utopian Dream or a Possible Reality?' with excellent national/international participants including Reshma Pai - President FOGSI (Elect) 2017, Madhu Chopra, Managing Director – Studio Aesthetique, Neerja Batle – Professor Department of Obstetrics and Gynecology, All India Institute of Medical Sciences, New Delhi, Christine Kaseba-Sata - Former First Lady, Republic of Zambia, Genevieve Sambhi – Former Miss Malayasia and a cervical cancer survivor, to name a few. Barkha Dutt reiterated that 65 countries have already accepted HPV vaccination program, adopted as a national program.

It is essential to remove any stigma associated with cervical cancer, and assure safety of the vaccine with no

serious side-effects in HPV naïve girls, is the critical information for all the stakeholders. The sessions on Scene Setting, Bringing Screening Services to Women, Global experiences in introducing Vaccines, Availability, Accessibility and Affordability of Treatment, set the tone for the India to battle cervical cancer. Dr. Dhananjaya Saranath highlighted the contribution of Cancer Patients Aid Association indicating a holistic approach and 'Total Management of Cancer', the vision and mission of CPAA. The focus of CPAA included – Cancer Awareness and Screening, Diagnosis, Patient Care, Research on Psycho-Social-Behavioral aspects of Cancer Patients and HPV molecular diagnostic tests, Affordable Cancer Insurance in conjunction with New India Assurance as partners, and rehabilitation for cancer survivors through 'CPAA Rehabilitation Centre' providing a modicum of economic/financial independence.

The take home message from the meeting deliberations were extremely optimistic emphasizing necessity of planned cervical cancer awareness with screening, treatment and follow-up. The meeting ended with the delegates committed to 'Cervical Cancer Screening and HPV Vaccination' in order to bring to reality 'Elimination (to zero) of Cervical Cancer'. The presence of manufacturers of quadrivalent HPV vaccine, assured their commitment to cervical cancer elimination, emphasizing priority to 'Women Health in India'. The role of media, National Radio/Television/Print and Digital Media support will ensure success of 'Women Health – Free of Cervical Cancer'.

Dr. Sankaranarayan, International Agency for Research in Cancer, Lyon, France, in his closing remarks appreciated the highly educative learning experience for all, the deliberations imparting a wealth of information. He summed up the comprehensive approach to prevention of cervical cancer in India, with the major recommendations and the road map as follows:

- Implementation of screening for women 30 – 65 years of age
- HPV Vaccination for all adolescent girls
- Mechanism of referral, treatment, management and palliative care
- Promotion of Research and Development towards new indigenous vaccines, and technologies to address cervical cancer diagnosis, prevention and

control
- Removal of associated stigma
- Awareness of Rights of women with respect to reproduction, sex and health
- Collaboration and Partnerships
- Sustainable financing
- Strengthening of health systems and generation of adequate trained workforce
- Engage with media and sensitization of medical professionals, scientists
- No female to be left behind'

The overreaching holistic impact of the meeting on women health was clear to all.

The current Biomedical Research Journal issue discusses an interesting theme of **Clusterin in cancer: A tumor suppressor gene or an oncogene?** by Dr. Tanuja Teni and Rajashree Kadam, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai. Clusterin (CLU), a molecular chaperone critical in cancer, lying at the cross road of life and death, as it functions as both an oncogene and a tumor suppressor gene in specific contexts, and hence a multifunctional gene. The contradictory functions of clusterin are reflected in promoting cell survival, activating autophagy and apoptosis, and on the other hand promoting tumor progression and inducing resistance to cancer treatment in vivo. This protein is ubiquitously expressed in diverse tissues and conserved across species, and is required to respond to exogenous or endogenous stress signals. Custirsen (OGX-011), a second generation antisense oligonucleotide sensitizes cancer cells to chemotherapy and radiotherapy, and in combination with HDAC-Inhibitor (Valproate) regresses tumor growth. Dr. Teni and Kadam, lucidly review the contrasting roles of CLU in cancer and associated regulatory mechanisms, highlighting Clusterin variants and functions.

The article on **Chemoprotectants in cancer chemotherapy: an update,** by Abhishek Basu, Arin Bhattacharjee, and Sudin Bhattacharya, Department of Cancer Chemoprevention, Chittaranjan National Cancer Institute, 37, S. P. Mukherjee Road, Kolkata, adds another dimension to cancer chemotherapy emphasizing use of chemoprotective agents to alleviate the toxic side effects of chemotherapeutic agents in cancer treatment. Chemotherapy is associated with significant toxicity and various

adverse impacting the outcome of treatment. The review highlights various US-FDA and several European regulatory agency approved chemoprotectants including amifostine, aprepitant, dexrazoxane, filgrastim, sargramostim, mesna, oprelvekin, palifermin, recombinant human erythropoietin, as well as indicate additional agents in cancer patient management. The authors point to the lacuna in the field in identification of novel, effective chemoprotectants.

In the same vein, we have Drs. Limbkar Kedar, Vaijayanti Kale and Lalita Limaye, from Stem Cell Laboratory, National Centre for Cell Science, NCCS complex, University of Pune Campus, Ganeshkhind, Pune, Maharashtra, give us a succinct article on recovery post irradiation on **Oral feeding with Arachidonic acid (AA) and Docosahexanoi acid (DHA) help in better recovery of haematopoiesis in sub-lethally irradiated mice.**

The authors experimentally depict the effect of polyunsaturated fatty acids (PUFAs) by oral administration of PUFAs-AA/DHA on haematopoiesis of sub-lethally irradiated mice in comparison to non-irradiated mice. The bone marrow cells of the mice were harvested and depletion was noted in the total nucleated cell (TNC) count, side population (SP) and $lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$(LSK) phenotype, and hemogram data of the PBCs. DHA or AA in the irradiated mice showed significantly higher number of BM-MNCs and increased percentage of SP and LSK cells, indicating better recovery and suggesting that DHA or AA may serve as useful dietary supplements in patients exposed to irradiation.

Mathematical modeling of viral epidemics: a review, by Pratip Shil, National Institute of Virology, Pashan, Pune, is an absolute must for all. Mathematical models to describe transmission and propagation of diseases have gained momentum particularly in the recent past with tremendous applications towards understanding the epidemiology of various diseases including viral diseases including Influenza, SARS, measles, bacterial disease such as tuberculosis, and drug resistant Staphylococcus. The advances in computational biology has enabled virtual simulations and mathematical modelling, particularly to understand the transmission routes and the epidemics/pandemics and facilitate informed decisive interventions and

vaccinations. Dr. Shil lucidly explains the various mathematical models and their applications in the study of virus driven epidemics.

Malaria which should have been a low incidence disease today, is still a sword of damocles in India and several countries, and hence the overview of **Recent advances in the treatment of malaria**, by Drs. Santosh R. Nandan, Evans Coutinho and their colleagues from Organics Pvt. Ltd. and Bombay College of Pharmacy, Mumbai, is timely. Malaria is a major cause of mortality and morbidity, and a well-developed treatment regimen including the artemisinins as well as safety preventive measures, have reduced the global burden of malaria in several countries. However, drug resistance is a developing problem in almost all infections including malaria. The authors focuses on clinical drug candidates with activity against several stages of the malarial parasite life cycle.

The final article on **Biomagnetic interaction of functionalized iron oxide nanoparticles with bovine serum albumin** by Dr. Sudeshna Chandra, Sunandan Divatia School of Science, NMIMS (Deemed-to-be) University, and Mr. Mayank Gupta, Department of Metallurgical Engineering and Materials Science, Indian Institute of Technology Bombay, Powai, Mumbai, highlight functionalized iron oxide (magnetic) nanoparticles as promising candidates for detection and sensing of target molecule. The study reports use of different macromolecules viz. glycol chitosan (GC), poly ethylene glycol methyl ether (PEGME) and poly sodium stereo-4 sulphate (PSSNa) to functionalize and cap magnetic nanoparticles. The magnetic nanoparticles were characterized and the structural and surface properties evaluated. Bovine serum albumin (BSA) was immobilized on the functionalized MNPs and using AC susceptibility studies the physical properties were measured.

The current issue of Biomedical Research Journal takes you from the doable today as seen by our 2016 Nobel Laureate Professor Yoshinori Ohsumi, to elimination of cervical cancer in India, the chemoprotectants and PFAs for better cancer patient management on chemotherapy and radiotherapy, to epidemiology and transmission studies by mathematical modelling, outlook into possible better therapy in malaria to the final contemporary topic of functionalized iron oxide nanoparticles.

Review

BRJ
Biomedical Research Journal

Clusterin in Cancer: Dual role as a Tumor Suppressor Gene and an Oncogene

Rajashree Kadam[1,2] and Tanuja Teni[1,2]*

[1]Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai – 410210, India
[2]Homi Bhabha National Institute, Training School Complex, Anushakti Nagar, Mumbai – 400085, India

Clusterin (CLU), a heterodimeric and sulfated glycoprotein has been associated with various physiological functions. This molecular chaperone protein is ubiquitously expressed in diverse tissues and conserved across species. Differences in subcellular localization and possible existence of different CLU isoforms may contribute to its functional diversity. Increased or decreased expression of CLU has been observed in several cancers versus normal tissues and hence its role in tumorigenesis is controversial. Evidences from several studies imply that CLU may have a dual role as a tumor suppressor gene or an oncogene depending on the signal and cellular context. CLU possibly exerts its oncogenic role by inhibiting apoptosis, activating autophagy and modulating several signaling pathways like IGF-1/IGFR, EGFR, NF-kB, PI3K/AKT, TGFβ and select miRNAs. CLU may exert its tumor suppressive effects by regulating cell cycle and inducing apoptosis. In cancer, loss of heterozygosity (LOH), copy number loss at CLU locus, epigenetic modifications and expression of select miRNAs may lead to the downregulation of CLU. Custirsen (OGX-011), a second generation antisense oligonucleotide that inhibits CLU expression and increases sensitivity of cancer cells to chemotherapeutic drugs, is currently in phase III clinical trials. CLU is an attractive target in several cancers, however for effective targeting, it is essential to know whether it acts as an oncogene or a tumor suppressor gene in a specific tissue/cellular context. The current review attempts to discuss the two contrasting roles of CLU in cancer and associated regulatory mechanisms. This review also sheds light on the complex CLU splice variants, the varied functional attributes supporting the dual roles in cancer and limitations of the CLU research that warrant attention.

INTRODUCTION

Clusterin (CLU), a ubiquitously present sulfated chaperone glycoprotein was first isolated from ram rete testis fluid where it was shown to elicit clustering of Sertoli cells and also of erythrocytes *in vitro* from several species leading to its nomenclature 'Clusterin' (Fritz *et al.,* 1983). Despite 33 years of immense efforts by researchers to understand the diverse functions of this multifaceted

Key words: Clusterin, cancer, tumor suppressor gene, oncogene, chaperone, stress.
***Corresponding Author:** Tanuja Teni, Scientific officer 'F', Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai – 410210, India.
Email: tteni@actrec.gov.in

protein CLU, it still remains an enigma. Since its discovery, several CLU homologues with different names and diverse physiological functions have been isolated from different species and tissues for example testosterone repressed prostate message protein 2 (TRPM2), sulfated glycoprotein 2 (SGP2), apolipoprotein J (ApoJ) and several others (Bettuzzi *et al*., 1989; de Silva *et al*., 1990; Léger *et al*., 1987). However "Clusterin (CLU)" is the acceptable name for all the above identified proteins.

In humans, the CLU gene (Fig. 1) encodes a mRNA of approximately 2 kb which directs the synthesis of a 449-amino acid primary polypeptide chain. CLU has been reported to be present in the body fluids of all vertebrates and is also one of the most abundant proteins (100-300ug/ml) found in human serum. Numerous biological functions have been associated with CLU including lipid transportation, membrane recycling, tissue differentiation and remodeling, cell–cell or cell-substratum interaction, cell proliferation, and cell death (Rosenberg *et al.,* 1995; Shannan *et al.,* 2006; Trougakos *et al.,* 2002; Wilson *et al.,* 2000). Altered expression of this important molecular chaperone CLU has been associated with aging, atherosclerosis, different neurological disorders including Alzheimers disease, cardiovascular and metabolic disorders and cancers of different origins. Diverse tissue specific distribution of CLU suggests that its expression is tightly regulated by different signaling pathways in normal and diseased conditions (Trougakos *et al.,* 2013).

In the light of new discoveries and information in the Clusterin field and the ongoing studies on the role of Clusterin in oral cancers in our laboratory, this review attempts to simplify and describe the CLU variants and the dual cell/tissue specific context dependent role of CLU as an oncogene or tumor suppressor gene in cancer and the constant challenges posed by this fascinating protein in understanding its complex role in cancer.

CLU Spliced Variants

The complexity and the low clarity on the existence of different CLU isoforms and its functions have challenged researchers for the past several years. Briefly, there are two major variants of CLU namely the predominant secretory form (sCLU) and intracellular forms which include the nuclear CLU (nCLU) and other non-secreted variants. These

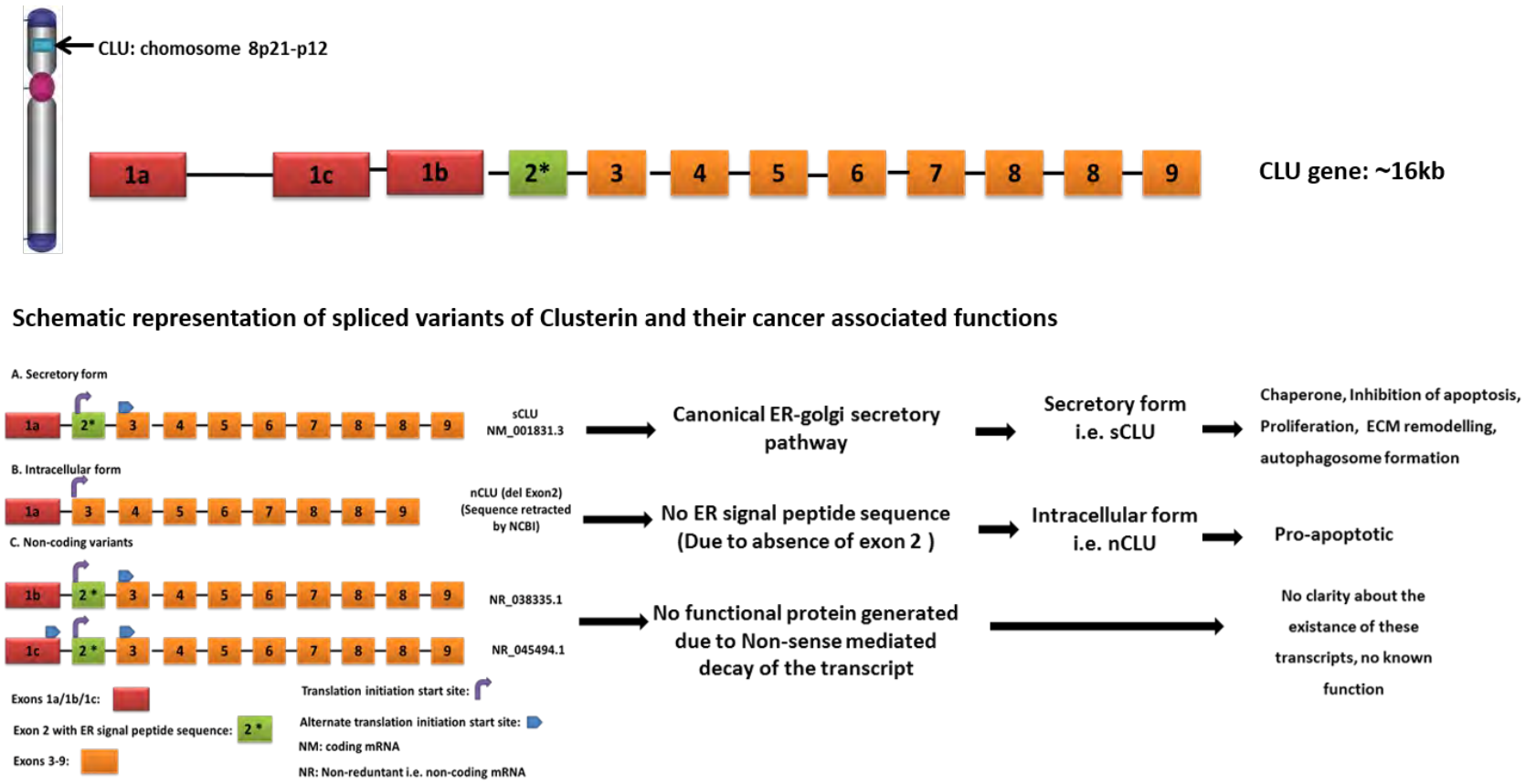


Figure 1: Schematic representation of spliced variants of Clusterin and their cancer related functions

CLU has following variants generated by alternate splicing event and differential use of exon 1:

A. Secretory form: Full-length variant generated by use of exon 1a

B. Nuclear form: N-terminally truncated variant generated by splicing of exon 1a to exon 3

C. Non-coding forms: These isoforms are predicted to use exon 1b and 1c, which do not code for functional protein due to nonsense mediated decay of these generated transcripts.

two isoforms have antagonistic functions i.e sCLU has prosurvival or antiapoptotic functions whereas nCLU has pro-death or pro apoptotic functions (Fig. 1) and are described below.

Secretory (extracellular) form i.e. sCLU (NM_001831.3)

This is the most predominant and commonly expressed anti-apoptotic isoform, synthesized as a full length secretory CLU via use of exon 1a and translation start site present upstream to signal peptide sequence on exon 2 (Prochnow *et al.,* 2013; Rizzi *et al.,* 2010). This signal peptide sequence of 22 amino acids encoded by exon 2 of CLU gene, directs the CLU protein to the ER where it undergoes N-linked glycosylation. Then this high mannose ER-precursor of 60kDa called pre secretory CLU (psCLU) enters the Golgi apparatus for further post translational modifications which include the addition of complex sugar moieties. The mature 80kDa CLU protein is further cleaved by a furin-like proprotein convertase which recognises the amino acid recognition motif RIVR to produce two polypeptide chains namely a N-terminal α-chain and C-terminal β-chain which are interlinked by five disulphide bonds thus yielding a heterodimeric mature secretory form (comprising of two sub units of 40 to 45kda each) (Jones *et al.,* 2002). Several groups have extensively studied the chaperone activity of sCLU.

The sCLU, a stress induced, ATP-independent extracellular chaperone protein is upregulated in several carcinomas like hepatocellular, lung, breast, bladder and in lymphoma, melanoma and downregulated in neuroblastoma, testicular seminoma and esophageal carcinomas (Chayka *et al.,* 2009; Koltai*,* 2014; Zhang *et al.,* 2003). It is not clear whether sCLU overexpression is a "cause" or "consequence" in the progression of a disease. Besides inducing proliferative and pro survival pathways as a signaling molecule, the cytoprotective role of sCLU is thought to be an outcome of the synergism of the chaperonic, scavenging and clearance activity of misfolded proteins and cellular debris. Different functional attributes of sCLU contributing to its pro-survival role in tumorigenesis are discussed further in detail, in this review.

Intracellular forms

In addition to the extracellular secretory form, several intracellular CLU forms

have been observed post stress and in damaged cells as described below.

nCLU (variant 1 del exon 2)

This putative nuclear pro-death form was initially demonstrated in MCF-7 breast cancer cell line and later on its occurrence was also demonstrated in prostate and colorectal carcinomas (Andersen *et al.,* 2007; Leskov *et al*., 2003; Rizzi *et al.,* 2010). This nCLU obtained by alternative splicing, generates N-terminally truncated isoform wherein exon 1 is spliced to exon 3 and thus lacks exon 2 bearing the ER signal peptide sequence, due to which the translation will initiate at the start site present on exon 3. Although the presence of three putative nuclear localization sequences (NLS) has been shown in nCLU, their presence was not found to be essential for its nuclear translocation (O'Sullivan *et al*., 2003). Interestingly, recent studies from our lab in oral cancer cell lines have demonstrated the localization of Clusterin in the nucleolus (unpublished data), which is a novel observation. Hence, whether nCLU is a different splice variant or is the sCLU which gets translocated to nucleus/nucleolus is not clear and warrants investigation. The nCLU has been shown to interact with Ku-70 of Ku-70/Ku-80 complex, thus impairing DNA repair and inducing apoptosis (Leskov *et al*., 2003). However, the sequence of nCLU is currently not available in NCBI database questioning the existence and the mechanism of nCLU transcript generation.

Stress induced intracellular non secreted CLU isoforms

Prochnow *et al*. (2013) demonstrated the generation of different CLU forms post stress and discussed the possible mechanisms for their generation: First they proposed that the post-translationally modified pre-mature CLU residing in endoplasmic reticulum is possibly re-translocated back to the cytoplasm. Secondly the authors proposed that the CLU transcript might use an alternative translation initiation site either present in exon 2, downstream to signal peptide sequence generating a truncated form of CLU or in exon 1, leading to a N-terminally elongated variant with a defect in the ER signal peptide sequence functionality, resulting in CLU accumulation in different intracellular organelles. Further these "non-secreted Clusterin isoforms" which are translated in negligible amounts

(about 0.34% of total CLU present in a cell) under stress conditions, possibly do not affect caspase 3/7 mediated apoptosis or NF-κB activity, thereby questioning their physiological relevance (Prochnow *et al.,* 2013). The only exception would be the hypoglycosylated form of CLU which interacts with GRP78, an ER stress associated protein which stabilizes the mitochondrial membrane, suggesting a possible role for CLU in unfolded protein response (UPR) and inhibition of apoptosis (Li *et al.,* 2013).

Non-coding/Non-redundant CLU isoforms

As shown in Fig. 1, these isoforms have been cited as Variant 2 (NR_038335.1) and variant 3 (NR_045494.1) in the NCBI database. These two variants are predicted to use exon 1b and 1c respectively and have been termed as "non-redundant or non-coding" isoforms as they do not code for a functional protein due to presence of an upstream ORF predicted to interfere with translation of the longest ORF due to which such a transcript generally undergoes nonsense mediated mRNA decay (NCBI database). Although variant 2 (NR_038335.1) is classified under non-coding isoforms, its presence was shown in the brain cells of Alzheimer's patients, suggesting a possible context dependent role for it which is yet to be explored (Ling *et al.,* 2012).

Thus, despite extensive efforts in the field of CLU research for the last several years, there is little clarity on the mechanism and regulation of different CLU transcript generation. As suggested by Essabbani *et al.* (2013), there might exist an "on demand alternative splicing" phenomenon generating the different isoforms in a context dependent manner.

Till date majority of the CLU research is focused on the prominent extracellular sCLU form and its chaperonic activities. One of the contributing factors for the low clarity on the existing CLU isoforms is the range of bands from 20-80kda obtained on a western blot following the use of different commercially available CLU antibodies. These bands are often found marked together as CLU in the antibody providing company data sheets. The development of CLU isoform specific antibodies may help to resolve the issue. However with the advent of new mass spectrometry based technologies it would now be possible to identify the different

forms of CLU seen on a gel and their post-translational modifications like glycosylation.

Structure of Clusterin

Despite the ubiquitous occurrence of extra and intracellular CLU forms and the ever increasing list of CLU interacting proteins, till date no crystallographic data is available for CLU. Several studies indicate that it has been very difficult to crystallize CLU protein due to its heavy glycosylation (almost 30% of the protein glycosylated) which is responsible for the "sticky" nature of this protein (Jones *et al.,* 2002). Also CLU exhibits a tendency to aggregate and form di, tetra and higher oligomers based on the pH, further adding to the difficulty in its crystallization. Hence majority of the available information on the secondary structure of CLU has been predicted through computational analysis, without any experimental support. sCLU exhibits a highly conserved primary structure across different species with highest homology displayed in the disulphide bonds and FC cleavage site (Bailey *et al.,* 2001).

Attempts have been made to characterize sCLU-client protein complexes using different techniques like size exclusion chromatography, dynamic light scattering, bis-ANS fluorescence spectroscopy, circular dichroism etc. These studies have shown the presence of 60% α-helices and also that CLU is likely to shield exposed hydrophobic regions of the client protein, resulting in the maintenance of secondary structure and stability of the same (Wyatt *et al.,* 2009). Further CLU structure has been predicted to be constituted of random coils and molten globule like regions as observed in proteins with ill-defined tertiary structure or in intrinsically disordered proteins like the heat shock protein family, essential for its chaperone functions. The amphipathic α-helical structure and intrinsically disordered molten globule structure attributes to its role as a "biological detergent", or scavenging/clearing agent which takes care of unfolded or undesired circulating macromolecules (Bailey *et al.,* 2001).

The sequence analysis of nCLU identified a conserved BH3 motif in its C-terminal coiled coil region (CC2) which interacts with Bcl2 family members as demonstrated by NMR analysis (Lee *et al.,* 2011). This is the only report till date which attempted to elucidate the interaction between nCLU

and Bcl2 family members using structural modeling and confirmed the proapoptotic function of nCLU by demonstrating its interaction with anti-apoptotic family members. Interestingly, the region of BH3 motif in CC2 region is common to both sCLU and nCLU, but it is the nCLU that interacts with Bcl2 family members and not the sCLU. Hence, it will be worth studying the interaction between sCLU and other BH3 motif containing family of proteins *in silico* which will help in understanding the basic CLU structure.

Functional aspects of Clusterin

Chaperonic functions of sCLU

sCLU was discovered as a molecular chaperone with extracellular activities like heat shock proteins and its expression is induced post stress via the CLE in its promoter. Through its chaperonic activity sCLU has been shown to play an important role in protein homeostasis in the cell to overcome stress conditions. sCLU prevents the aggregation of denatured proteins by binding to it in an ATP independent manner and forming high molecular weight soluble complexes (Rohne *et al.*, 2014). *In vitro* studies have demonstrated that sCLU facilitates uptake of these complexes in neighboring tissue cells for removal by lysosomes. sCLU interacts with scavenger receptors and contributes to removal of toxins in liver and kidneys. Interestingly studies demonstrate that the disulphide bonds of CLU are important for its maturation and correct folding but not for its chaperonic function. Similarly its glycosylation was demonstrated to be important for its correct polar secretion in cells but not for its chaperonic activity (Rohne *et al*, 2016).

Role for CLU in Phagocytosis

Interestingly another novel function of CLU as an opsonin in a process of efferocytosis i.e. phagocytosis of dying cell has been shown, suggesting a protective role for CLU in modulating immune response. CLU has been shown to bind on the blebs on late apoptotic cells and to histones accumulated in the cytoplasm of dying cells, which marks the cell for phagocytosis (Cunin *et al*., 2016). Another novel role of CLU in the clearance of excess of misfolded proteins has been reported in idiopathic pulmonary fibrosis (IPF), a lung disorder where excess of extracellular matrix gets accumulated. In this IPF condition, CLU has been shown to be downregulated,

which acts as a quality control regulator by binding to such misfolded proteins and promoting the phagocytosis process. In CLU-/- mice, impaired collagen/ECM clearance by macrophage driven phagocytosis has been demonstrated (Bernard *et al.,* 2015).

Role for CLU in Senescence

Recently the role of CLU in senescence was demonstrated. CLU has been shown to be transcriptionally up-regulated during both replicative senescence (RS) and stress induced premature senescence (SIPS). This upregulation of CLU occurs through the ATM/IGF-1/IGF-1R/MAPK/ERK-1/2/EGR-1 signaling pathway, which also overlaps with DNA damage response (DDR) pathway. Earlier it was deciphered that as sCLU is an anti-apoptotic protein, it may cause population doubling thereby preventing cell death. However knockdown of sCLU in middle aged and senescent cells did not exhibit apoptosis, suggesting that the anti-apoptotic function of sCLU may not be operative during senescence (Luo *et al.,* 2014).

CLU knockout studies

CLU knockout studies revealed that CLU knockout mice were fertile and had no obvious phenotype (Rosenberg *et al.,* 1995). Also mice development was not affected by the absence of CLU. However, these mice showed increased sensitivity to autoimmune myocarditis, suggesting a role for CLU in protecting the heart tissue from post inflammatory destruction. CLU-/- mice exhibited severe inflammation and changes in cellular pathology in experimentally induced murine autoimmune myocarditis as compared to CLU-expressing control mice (McLaughlin *et al.,* 2000). In contrast in another study, in the absence of CLU, mice were found to be partially protected after hypoxic injury, suggesting that CLU appears to have a negative role in neuronal survival (Han *et al.,* 2001).

$CLU^{-/-}$ mice showed impaired morphogenic and functional features of regenerating pancreas. These mice exhibited loss of regenerating capacity of the beta cells resulting in a hyperglycemic condition, implying a role for Clusterin in promoting regeneration following pancreas injury and in *in vitro* beta-cell regeneration (Lee *et al.,* 2011). Studies demonstrated that damage to testicular cells is increased after heat shock in $CLU^{-/-}$ mice and additionally the clearance of damaged cells is also impaired (Bailey *et al.,* 2002). Further, in

ageing $CLU^{-/-}$ mice, progressive glomerulopathy characterized by accumulation of insoluble protein deposits in kidneys was observed indicating that CLU may inhibit age-dependent accumulation of protein deposits in the glomeruli (Rosenberg *et al.,* 2002).

Role of CLU in tumorigenesis

Over the past 15 years a significant amount of data has been generated on CLU expression in different tumor tissues, however the discrepancy of its role in cancer still prevails. Overexpression of CLU in some cancers indicates its role as an oncogene, while its repression or downregulation in other cancers conversely indicates that it may have a tumor suppressive function. This review is an attempt to conciliate and address the available information on Clusterin's apparently contradictory and possibly context dependent and tissue specific role in cancer.

Evidence for Clusterin as a tumor suppressor gene

The first *in vivo* evidence for the possible role of CLU as a tumor suppressor came from the work by Thomas-Tikhonenko *et al*., 2004 which demonstrates that CLU-null mice are prone to development of skin cancers. Further studies by Davoli *et al.* (2009) demonstrated that siRNA mediated knockdown of sCLU leads to cell cycle progression with increase in proliferation markers. Additional support for the tumor suppressor function of CLU was provided by the TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice which exhibited aggressive tumor development when crossed to CLU-/- mice due to inactivation of one or both *CLU* alleles in TRAMP mice. Interestingly the TRAMP/CluKo mice exhibited enhanced tumor spreading and homing, early metastases in ectopic sites and decreased survival. Further 30% of these mice died by 28 weeks versus none of the TRAMP only group. These studies thus suggest CLU to be a negative modulator of prostate cancer and a putative haploinsufficient tumor suppressor gene.

Studies by Chayka *et al.* (2009) demonstrated that CLU acts as a negative modulator of growth in neuroblastoma. The authors showed that MYCN amplification via the activation of miR17-92 cluster brings about sCLU suppression. Intriguingly the penetrance of neuroblastomas arising in MYCN-transgenic mice was significantly

increased after deletion of the CLU gene, suggesting it to be a tumor suppressor protein. Further confirmation for this came from the studies showing that sCLU siRNA-transduced neuroblastoma cells exhibited increased metastases when xenografted in mice with concomitant activation of NF-ƙB signaling and epithelial to mesenchymal transition (EMT).

Andersen *et al.* (2007) reported the downregulation of CLU isoforms in colorectal carcinoma (CRC). Using genome-wide analysis they showed LOH and concomitant copy number loss at the CLU locus 8p21 in 67% CRC cases. Further analysis revealed that TCF1-mediated Wnt-signaling along with loss of copy number at CLU locus is responsible for the observed CLU downregulation (Schepeler *et al.,* 2007). CLU expression was also reported to be significantly lower in testicular seminoma as compared to normal testis. Testicular seminomas are one of the most sensitive tumors being responsive to radiotherapy and chemotherapy. This further supports the role of sCLU as a cytoprotective protein, protecting cells from death due to anti-tumor therapy (Liu *et al.,* 2013). Studies carried out by Chen *et al*. (2014) to identify host immune response protein candidates in the sera of oral squamous cell carcinoma patients, revealed that CLU is one of the downregulated genes. Preliminary data from our lab have demonstrated downregulation of sCLU in oral tumor tissues as compared to normal oral mucosa. Studies are ongoing to elucidate the mechanism of CLU downregulation and its role in oral cancers.

Clusterin-positive patients with pancreatic cancer exhibited significantly longer survival as compared to Clusterin-negative patients indicating that down-regulation of CLU may be involved in the progression of pancreatic cancer (Xie *et al*., 2002). However this observation is not consistent with current reports where Clusterin has been shown to confer chemoresistance in pancreatic cancers suggesting a role as an oncogene (Kong *et al.*, 2012; Tang *et al.,* 2012). Such contradictory reports add to the complexity of the subject and the dilemma whether CLU is a tumor suppressor or an oncogene.

The following functions/regulation of sCLU might attribute to its tumor suppressive functions/role.

Epigenetic regulation of CLU expression

Several evidences suggest that regulation of CLU expression at genomic level is effected through either epigenetic mechanism or large- scale deletion of the gene. Rat fibroblasts transformed with Ha-Ras exhibited downregulation of Clusterin mediated by deacetylation of CLU promoter followed by methylation via the MEK/ERK signaling pathway (Lund *et al.,* 2006). Earlier reports have demonstrated that CpG island methylation or histone deacetylation in the proximity of the *CLU* gene leads to the downregulation of Clusterin in neuronal cells, tumor endothelial cells and prostate cancer (Hellebrekers *et al*., 2007; Nuutinen *et al*., 2005; Rauhala *et al*., 2008). Another report in hepatocellular carcinoma demonstrated regulation of CLU through acetylation/ deacetylation of histone H3 within the CLU promoter (Liao *et al.,* 2009). In 2014, Park *et al.* (2014) studied the transcriptional regulation of nCLU in response to hypoxia, where binding of HIF1-α to the three putative hypoxia responsive elements (HREs) was shown, to induce nCLU expression followed by apoptosis in prostate cancer cell line PC3, but not in LNCaP cells. Further analysis revealed that *CLU* promoter was not methylated in PC3 cells; but was methylated in LNCaP cells suggesting that nCLU expression is regulated by direct binding of HIF-1α to HRE sites and is epigenetically controlled by methylation of its promoter region. Similar studies in breast carcinoma demonstrated absence of CLU expression in normal breast tissue due to methylation of CLU promoter, while in breast carcinoma tissues CLU promoter was found to be demethylated resulting in its overexpression (Serrano *et al.,* 2009). Recently, Amente *et al*. (2015) demonstrated that MYCN mediated downregulation of CLU was a result of the interaction of MYCN with lysine specific demethylase-1 (LSD1), which has been shown to be essential for repression of CLU gene expression.

Regulation of CLU by microRNAs

miRNAs are small (~ 22 nucleotides), non-coding single stranded RNA molecules involved in post-transcriptional gene regulation, by binding to the 3'-UTR region of targeted mRNA. These miRNAs act generally in a context dependent manner either as an oncogene or tumor suppressive miRNA (Erhard *et al*., 2014).

In neuroblastoma, Chayka *et al.* (2009) demonstrated that, CLU is negatively regulated by the protooncogene MYCN through the activation of the miR 17-92 cluster. This was further supported by a report which showed that the expression of two microRNAs in that cluster, miR-17-5p and miR-92, is upregulated by MYCN expression in SH-EP neuroblastoma cells. Further analysis using miRanda, a web based algorithm revealed that CLU mRNA was a target for miR-17, miR-18a and miR-19a which is known to be induced by c-MYC in a human B-cell line. However further validation using luciferase assay and miR mimics could not demonstrate direct binding of these miRs to the 3'UTR region of CLU, suggesting that it might possibly target some upstream CLU activator, thereby downregulating CLU expression (Sala *et al*., 2009).

Different miRNA microarray studies have revealed the overexpression of miR-21 in head and neck squamous cell carcinoma (HNSCC) (Shiiba *et al*., 2010) and further studies have indicated CLU to be potential target of miR-21. CLU was found to be downregulated following the expression of miRNA-21 in normal and HNSCC cell lines and tissues, thereby modulating cell growth properties (Mydlarz *et al.,* 2014). These reports suggest that miRNAs may have a key role in regulating CLU levels, defining the tumor suppressive function of CLU in a context dependent manner.

Modulation of NF-kB pathway by CLU

In 2003, Santilli *et al.* (2003) demonstrated that transfection of CLU in both normal and tumourigenic cells (LAN5 neuroblastoma cell line) caused stabilisation of NF-kB inhibitors, resulting in inhibition of NF-κB activity. Further, Devauchelle *et al*. (2006) demonstrated that CLU interacted with phosphorylated IkBα to prevent E3 ubiquitin ligase binding leading to IkBα stabilization, thereby preventing NF-kB translocation to the nucleus, thus implying CLU to be a negative modulator of NF- kB activity.

Evidence for Clusterin as an oncogene

Tumor cell survival and progression has been shown to be associated with increased levels of intracellular and secretory forms of CLU. The ability of CLU to function as an oncogene is mainly attributed by its ability to promote cell growth and inhibit apoptosis. Within the cell, sCLU blocks

apoptosis by binding to ku70-Bax complex, as a cytosolic retention factor and preventing its translocation to the mitochondria (Trougakos *et al.,* 2009). This interaction obstructs Bax oligomerization, which does not allow the release of cytochrome *c* from mitochondria and caspase activation. Further, sCLU was shown to inhibit the oncogenic c-Myc-induced apoptosis by interacting with conformation-altered Bax (Zhang *et al.,* 2005). Recently the role of CLU in prosurvival autophagy has been demonstrated where CLU was shown to interact with LC-3 via LIR-binding sequence within autophagosome membrane, which causes LC-3 lipidation and facilitates LC-3 and Atg-3 complex stabilization leading to autophagy initiation. In CLU-/- mice and prostate cancer cells with CLU knockdown, autophagy was shown to be attenuated, suggesting a role for CLU in pro-survival autophagy (Zhang *et al.,* 2014).

Sensibar *et al.* (1995) demonstrated the role of SGP-2/ sCLU in the prevention of cell death induced by TNF-α in LNCaP prostate cancer cell line. The high expression of CLU in renal cancer cells was significantly associated with pathological stage and grade of the tumor, and with poor overall and recurrence-free survival rate of patients (Miyake *et al*. 2002a). There are several indirect evidences in the literature which suggests that sCLU is an oncoprotein. Studies have shown that CLU silencing affected the chemosensitivity of human pancreatic cells to gemcitabine by either modulating NF-ƙB activity or inhibiting clusterin-dependent pERK1/2 activation (Kong *et al*., 2012; Tang *et al*., 2012). Further, over-expression of CLU in transitional cell carcinoma of the bladder was shown to prolong cell survival, resulting in enhanced metastatic potential *in vivo*, indicating its possible use as a marker for prognosis and tumor recurrence (Miyake *et al.,* 2002b).

Another evidence for the role of CLU in oncogenesis came from the studies by Chou *et al*. (2009) in lung adenocarcinoma, where its role in epithelial to mesenchymal transition was demonstrated and CLU was shown to be a positive indicator of the degree of invasiveness in lung adenocarcinoma cell lines. CLU silencing resulted in mesenchymal to epithelial transition (MET) as evidenced by the spindle-to-cuboidal morphological change, increased E-cadherin expression, and decreased fibronectin expression. The levels of slug protein, a zinc finger

containing transcription factor that represses E-cadherin, were reduced in the CLU silenced cell lines. Also the ERK levels correlated with that of slug and CLU. These studies indicate a role for Clusterin in EMT and ERK/Slug signaling. Overexpression of CLU and its role in invasiveness has been reported in laryngeal squamous cell carcinoma wherein siRNA knockdown of CLU was found to inhibit cell proliferation and induce apoptosis *in vitro* (Wang *et al.,* 2014). Studies demonstrate that B-MYB binds to and positively regulates the CLU promoter through a MYB-consensus element. In fibroblasts transfected with a dominant-negative B-MYB construct, which suppressed the thermal induction of CLU, thermal injury was prominently observed. B-MYB induced CLU has also been shown to confer doxorubicin resistance in human LAN5 neuroblastoma cells (Cervellera *et al.,* 2000; Santilli *et al.,* 2005).

Role of CLU in the recruitment of monocyte/macrophage infiltration at the tumor site and its role in invasion were studied by Shim *et al*. (2011). In monocytes and macrophages, CLU was shown to regulate MMP-9 expression via ERK1/2 and PI3K/AKT/NF-κB pathways, which contribute to the tissue reorganization by serving as a modulator for extracellular matrix degradation. Further CLU facilitated IκB degradation by SCF complex (E3 ubiquitin ligase complex) and nuclear translocation of NF-κB p65 (Zoubeidi *et al*., 2010) which is critical for MMP-9 expression. Thus CLU provides connecting link between two cellular processes i.e. inflammation and cancer by increasing NF-κB and MMP-9 levels. Recently, Li *et al.* (2016) have shown that CLU is induced by N, N'-dinitrosopiperazine (DNP), a known carcinogen responsible for the development of nasopharyngeal carcinoma (NPC). It was shown that post-DNP treatment, CLU, VEGF and MMP-9 levels increases and interestingly increase in VEGF and MMP-9 was via increased CLU expression. CLU was shown to interact with VEGF and MMP-9, which was responsible for invasiveness and metastasis.

These pro-survival functions of sCLU might attribute to its oncogenic function, role in other diseased conditions, and also to the increased resistance of cancer cells to different chemotherapeutic agents, like doxorubicin, cisplatin and taxol (Djeu *et al.,* 2009). This is evident from the observation that depletion of sCLU by

antisense or small interfering RNA caused hypersensitization of cancer cells to paclitaxel or IR (Criswell *et al.,* 2005; So *et al.,* 2005).

CLU induction via regulatory pathways

The complex mechanism of transcriptional regulation of CLU gene and the existence of more than one regulatory promoter region may be responsible for the varied expression pattern of CLU proteins. Studies by Wong *et al.* (1994) revealed that the proximal promoter region of CLU (P1) showed presence of different cis-regulatory elements including AP-1, AP-2, and SP-1 motifs. Additionally, a long domain of 14bp conserved among different species called as Clusterin element (CLE), was found to be related to heat-shock response element (HSE), which differed by just a single base. Further, another putative promoter region located in intron 1 of CLU (P2) was predicted to have a TATA box, cAMP responsive element (CRE) and CAAT box sequences. These predicted regulatory elements present in the promoter region of CLU may possibly have a role in the regulation of CLU in a context dependent manner, which needs to be validated experimentally.

The different regulatory pathways involved in CLU induction are described below and illustrated in Fig. 2.

NF- kB pathway

Zoubeidi *et al*., 2010 showed that, CLU facilitated degradation of inhibitors of NF- ƙB i.e. IkB and Copper metabolism gene MURR1 domain-containing protein (COMMD1) in response to different cellular stress by SCF E3 ubiquitin ligase complex, thereby enhancing NFκB activity in prostate cancer cell line (Fig. 2A). Thus, NF-κB induces further sCLU expression turning on a positive feedback loop.

TGF-β signaling

The TGF-β signaling pathway also plays a key role in sCLU induction via activation of transcription factors like AP-1 and EGR-1 which are well documented to activate sCLU transcription. TGF- β signaling has also been shown to induce de-repression of sCLU transcription mediated by c-FOS (Jin and Howe, 1999). sCLU has been shown to bind to both TGF- β type-I and II receptors by yeast two-hybrid screening and transmit signaling via the conventional pathway. TGF- β treatment

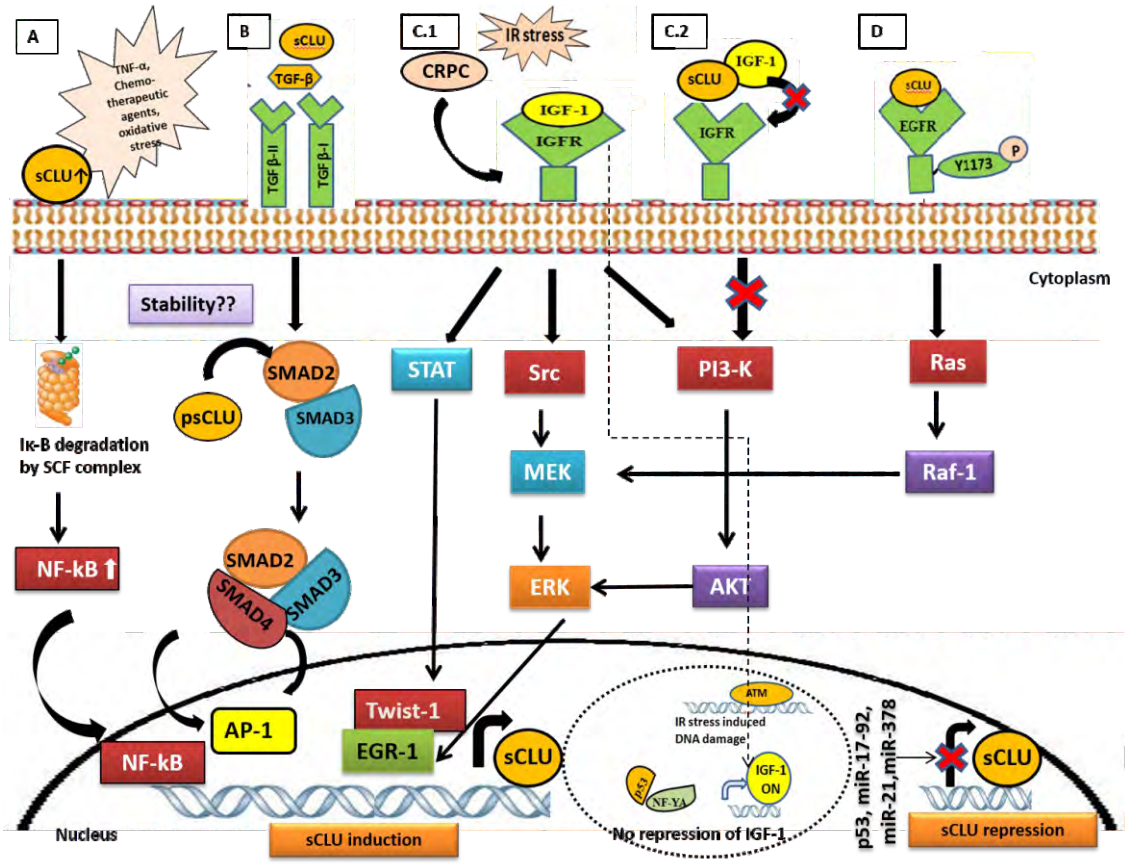


Figure 2: Schematic representation showing different regulatory pathways involved in sCLU induction
sCLU has been shown to bind to different receptors on the cell membrane, activating different cellular pathways. A) Under stress conditions like increase in TNF-α, chemotherapy etc. sCLU levels increases which causes degradation of NF-kB inhibitors, activating this pathway. B) sCLU can also bind to both TGF-beta receptors and can activate the pathway mediated by SMAD2/3 and SMAD4 complex. psCLU binds to SMAD2/3 intracellularly, maintaining their stability probably by preventing their proteasomal degradation. C.1) In different stress conditions like IR exposure, DNA damage induced ATM is activated which causes de-repression of IGF-1 mediated by p53-NF-YA complex. This activates pro-survival pathway i.e. IGF-1/IGF-1R which in turn activates MEK/ERK pathway leading to activation of EGR-1, a well-known transcription factor known to activate sCLU transcription. C.2) IGF-1 binding to IGF-1R can also activate PI3K/AKT pathway, which is blocked by binding of sCLU to IGF-1 extracellularly. D) sCLU binds to EGFR and activates Ras dependent Raf-1/MEK/ERK pathway.

causes translocation of CLU from the cytoplasm to nucleus in the HepG2 and CCL64 epithelial cell lines (Reddy *et al.,* 1996). psCLU has been shown to modulate the stability of SMAD2/3 by binding to it intracellularly. Thus the overexpression of CLU enhanced TGF- β induced transcriptional activity resulted in increased amounts of Smad2/3 proteins (Fig. 2B). This increased stability of Smad2/3 is not due to direct binding of CLU to Smad2/3; but because CLU possibly prevents the proteasome mediated degradation of Smad2/3 (Lee *et al.,* 2008). Recently a role for CLU as a mediator of the TGF-β induced epithelial

to mesenchymal transition (EMT) was demonstrated. Studies revealed that Twist-1 mediated TGF-β-induced CLU expression by binding to E-box elements in the distal promoter region of CLU gene (Shiota *et al.,* 2012).

IGF-1/IGF-1R signaling

It is well documented that CLU is induced post treatment with low non-toxic doses of IR (0.02-0.5 Gy), suggesting a role for CLU in radiation adaptive responses, characterized by increased radioresistance. Survival of damaged cells after IR leads to genomic instability (Klokov *et al.,* 2004). IGF-1/IGF-1R signaling pathway is one of the most common pro-survival pathway constitutively upregulated in several types of cancer. Studies to investigate whether sCLU induction occurs via this pathway revealed that IR stress induced DNA damage causes activation of Ataxia telangiectasia-mutated kinase (ATM), which causes de-repression of IGF-1 transcription mediated by p53-NF-YA complex. As a result of this IGF-1 levels increase leading to the activation of IGF-1/IGF-1R pathway which further activates downstream targets like Src MEK/ERK or PI3K/AKT (Ammar and Closset, 2008; Zhang *et al.,* 2014) which inturn activates EGR-1 transcription factor and further induction of sCLU transcription (Figs. 2C.1 and 2C.2) (Goetz *et al.,* 2011). This provides a connecting link between p53 mediated suppression of sCLU post IR induction and IGF-1/IGF-1R signaling (Criswell *et al.,* 2005).

Interestingly under stress conditions like serum deprivation, sCLU has been shown to bind to and sequester IGF-1 extracellularly, to prevent IGF-1 binding to IGF-1R, thus negatively modulating the PI3K-AKT pathway (Jo *et al.,* 2008). In hepatocellular carcinoma, high expression of CLU has been shown to be associated with poor survival and high tumor recurrence, wherein CLU overexpression has been shown to activate PI3K/AKT pathway by interacting with EIF3I, leading to the further activation of MMP13 and to metastasis. Interestingly knockdown of CLU was shown to affect the CLU-EIF3I/AKT/MMP13 axis, suppressing metastasis (Lee *et al.,* 2016). CLU is overexpressed in castration resistant prostate cancer (CRPC) where the pro-survival pathway like IGF-1/IGF-1R pathway is well studied wherein sCLU

has been shown to be induced via the STAT-Twist-1 signaling in this pathway (Takeuchi *et al.,* 2014).

EGFR pathway

Studies by Shim *et al.* (2009) suggest a role for CLU in astrogliosis or reactive astrocytosis in which an abnormal increase in the number of astrocytes occurs due to loss of nearby neurons caused by accidental injury, ischemia, autoimmune disorder or neurodegenrative disorders, mediated via the EGFR pathway. Their studies revealed that sCLU binds to epidermal growth factor receptor (EGFR), transmitting mitogenic signal downstream via the Ras dependent Raf/MEK/ERK pathway in rat astrocytes (Fig. 2D). It is not known whether the activated ERK further activates EGR-1(early growth response-1), a well-documented transcription factor for sCLU transcription, leading to a positive feedback loop inducing cell growth and proliferation.

Regulation of CLU by miRNA

In non-small cell lung carcinoma (NSCLC), CLU has been shown to be upregulated and confer resistance to chemotherapeutic agents like cisplatin. Recently, miR-378 has been shown to target CLU, which chemosensitizes NSCLC cells highlighting its therapeutic importance (Chen *et al.,* 2016).

From the above information, it is still unclear whether the opposing functions of CLU reported in the literature are due to the use of different antibodies by different groups, the lack of antibodies specifically recognizing different forms of CLU, the type of cell lines, patients, etc studied or whether it indicates that CLU can act as a tumor suppressor or oncogene, depending on the type of cancer and its phase of progression. It is possible that the prominent role of CLU in the different normal tissues may be a determining factor of its role as a tumor suppressor gene or oncogene in the malignant tissues.

Targeting CLU for treatment of advanced cancers

In majority of the cancers, the conventional treatment modalities include surgery, chemotherapy, radiotherapy and alternatively in case of prostate and breast cancers, hormone ablation therapy. Overall, about one third of the cancer patients show recurrence and resistance to different anti-cancer therapeutics. One of the important

contributing factors for this development of resistance would be overexpression of certain pro-survival factors including stress induced cytoprotective chaperonic sCLU, which is upregulated in several cancers as mentioned earlier in this review. It has been speculated that sCLU might confer resistance to the different therapies by modulating several cellular processes like apoptosis, cell cycle checkpoints, inflammation etc. Hence, targeting sCLU may help to improve the efficacy of current therapeutic strategies by sensitizing the cancer cells to the different therapeutic agents.

Custirsen (OGX-011), is a second generation anti-sense oligonucleotide (ASO) designed by OncoGeneX Technologies Inc. in collaboration with Isis Pharmaceuticals and is directed against the translation start site located in exon 2 of sCLU. ASO comprise of chemically modified stretch of DNA that targets specific mRNA, and further inhibits its translation by forming DNA/RNA duplex. However, a major disadvantage of using ASO is its instability and rapid intracellular degeneration. Custirsen is a phosphorothioate antisense oligo-nucleotide, which also has the 2'-MOE modification on the 4 bases on either end of the 21-mer phosphorothioate backbone. This ASO to CLU exhibited a significantly higher affinity for the target and better potency in terms of its increased half-life (7 days) and longer duration of its action as compared to first generation ASOs (Zellweger *et al.,* 2001). In a phase I clinical trial aimed to study the pharmacokinetics and pharmacodynamics of OGX-011 and its efficacy in treatment of patients with localized prostate cancer revealed that OGX-011 can be safely administered to humans at a dose of 640 mg (Chi *et al.,* 2008). Further studies have shown that OGX-011 improved the efficacy of radiatiotherapy, chemotherapy and hormone ablation therapy by inhibiting sCLU expression and enhancing apoptosis (Koltai *et al.,* 2014). Studies by Trembley *et al*. (2013), (Patent no.: WO 2013123588 A1) showed that co-targeting CLU and EGFR using their respective inhibitors i.e. h16B5 and Erlotinib is a promising strategy in non-small cell lung carcinoma (NSCLC) and prostate cancer patients

Concluding remarks

CLU, a stress-induced multifunctional glycoprotein is vital for maintaining cellular homeostasis, predominantly via

its role as a chaperone. Based on the available information in the literature, there is little clarity on the CLU isoforms and their functions in cancer and research is warranted in this area to decipher the same. The potentially conflicting evidence of overexpression and repression of CLU in different cancer tissues suggests a dual role for CLU as a tumor suppressor or an oncogene. The mechanism of CLU regulation is signal and cellular context dependent, deciphering which is a challenge. Although the existence of a nuclear CLU is controversial, the possible occurrence of hypoglycosylated and glycosylated forms with opposing functions and differential localization is speculated and may support its tumor suppressive and oncogene roles. Development of an antibody that distinguishes these two forms of CLU and deciphering its crystal structure may help in clarifying the dual role of CLU.

The complex role of CLU in cancer is far from being resolved. However with the advent of new technologies, it may be possible to gain some clarity in the role of CLU variants in cancer. Using high end mass spectrometry techniques, it may be possible to identify the different CLU variants detected post stress, in different types of tumors and cell lines. However the identification of these variants can be further strengthened by the development of variant specific antibodies for their antibody-based detection in the cells and tumors. Also, clarity on the functions of CLU variants in a specific cancer tissue can be obtained by performing knockdown/knockout studies of specific CLU variant and followed by rescue experiments. Using latest molecular imaging techniques, the route and destination of the labeled CLU proteins can be tracked in cancer versus normal cells to understand their cellular function. Identification of the sCLU interactome in normal versus tumor tissues will provide clues to its binding partners and possible functions in these tissues. High CLU expression has been associated with tumor progression, therapy resistance and poor prognosis and studies indicate that CLU can serve as a biomarker/predictor of response post drug treatment. However, caution needs to be exercised in the use of CLU ASO- Custirsen to target CLU in cancer and it would be important to ascertain whether CLU is a positive or negative modulator of carcinogenesis in the specific cancer tissue.

REFERENCES

Amente S, Milazzo G, Sorrentino M, Ambrosio S, Di Palo G, Lania L, *et al.* Lysine-specific demethylase (LSD1/KDM1A) and MYCN cooperatively repress tumor suppressor genes in neuroblastoma. *Oncotarget* 2015;*6*(16):14572–14583.

Ammar H, Closset JL. Clusterin activates survival through the Phosphatidylinositol 3-Kinase/Akt pathway. *J Biol Chem* 2008;283:12851–12861.

Andersen CL, Schepeler T, Thorsen K, Birkenkamp-Demtröder K, Mansilla F, Aaltonen LA, *et al.* Clusterin expression in normal mucosa and colorectal cancer. *Molecular & Cellular Proteomics* 2007; 6:1039–1048.

Bailey RW, Dunker AK, Brown CJ, Garner EC, Griswold MD. Clusterin, a binding protein with a molten globule-like region. *Biochemistry* 2001;40:11828–11840.

Bailey RW, Aronow B, Harmony JA, Griswold MD. Heat shock-initiated apoptosis is accelerated and removal of damaged cells is delayed in the testis of clusterin/apoJ knock-out mice. *Biol Reprod* 2002; 66:1042–1053.

Bernard K, Kurundkar D, Wang Y, Deshane J, Thannickal V. Clusterin deficiency promotes persistent fibrosis by impairing phagocytic clearance of collagen and promoting myofibroblast survival. *Am J Respir Crit Care Med* 2015;191:A6095

Bettuzzi S, Hiipakka RA, Gilna P, Liao ST. Identification of an androgen-repressed mRNA in rat ventral prostate as coding for sulphated glycoprotein 2 by cDNA cloning and sequence analysis. *Biochem J* 1989;257:293–300.

Cervellera M, Raschella G, Santilli G, Tanno B, Ventura A, Mancini C, *et al*. Direct transactivation of the anti-apoptotic gene Apolipoprotein J (Clusterin) by B-MYB. *J Biol Chem* 2000; 275:21055–21060.

Chayka O, Corvetta D, Dews M, Caccamo AE, Piotrowska I, Santilli G, *et al*. Clusterin, a haploinsufficient tumor suppressor gene in neuroblastomas. *J Natl Cancer Inst* 2009;101:663–677.

Chen Y, Azman SN, Kerishnan JP, Zain RB, Chen YN, Wong Y-L, et al. Identification of Host-immune response protein candidates in the sera of human oral squamous cell carcinoma patients. PLoS ONE 2014; 9(10): e109012.

Chen X, Jiang Y, Huang Z, Li D, Chen X, Cao M, *et al*. miRNA-378 reverses chemoresistance to cisplatin in lung adenocarcinoma cells by targeting secreted clusterin. *Scientific Reports*. 2016; 6:19455.

Chi KN, Siu LL, Hirte H, Hotte S, Knox J, Kollmansberger C, *et al*. A phase I study of OGX-011, a 2-methoxyethyl phosphorothioate antisense to clusterin, in combination with docetaxel in patients with advanced cancer. Clin Cancer Res 2008;14:833–839.

Chou TY, Chen WC, Lee AC, Hung SM, Shih NY, Chen MY. Clusterin silencing in human lung adenocarcinoma cells induces a mesenchymal-to-epithelial transition through modulating the ERK/Slug pathway. *Cellular Signaling* 2009; 21:704–711.

Criswell T, Beman M, Araki S, Leskov K, Cataldo E, Mayo LD, Boothman DA. Delayed activation of insulin-like growth factor-1 receptor/Src/MAPK/Egr-1 signaling

regulates clusterin expression, a pro-survival factor. *J Biol Chem* 2005; 280(14):14212–14221.

Cunin P, Beauvillain C, Miot C, Augusto J, Preisser L, Blanchard S, Pignon P *et al.* Clusterin facilitates apoptotic cell clearance and prevents apoptotic cell-induced autoimmune responses. *Cell Death and Disease* 2016; 7, e2215.

Davoli S, Davalli P, Chayka O, Rizzi F, Pellacani D, Fregni G, *et al*. Effects of Clusterin knockdown on prostate cancer progression in the TRAMP model. *The FEBS Journal* 2009; 276:363–364.

de Silva HV, Stuart WD, Park YB, Mao SJ, Gil CM, Wetterau JR, *et al*. Purification and characterization of apolipoprotein J. *J Biol Chem* 1990; 265:14292–14297.

Djeu JY, Wei S. Clusterin and Chemoresistance. *Adv Cancer Res* 2009; 105:77–92.

Devauchelle V, Essabbani A, De Pinieux G, Germain S, Tourneur L, Mistou S, *et al.* Characterization and functional consequences of underexpression of clusterin in rheumatoid arthritis. *J Immunol* 2006;177(9):6471–6479.

Erhard F, Haas J, Lieber D, Malterer G, Jaskiewicz L, Zavolan M, *et al*. Widespread context dependency of microRNA-mediated regulation. *Genome Research* 2014;24(6):906–919.

Essabbani A, Garcia L, Zonetti MJ, Fisco T, Pucci S, Chiocchia G. Exon-skipping strategy by ratio modulation between cytoprotective versus pro-apoptotic clusterin forms increased sensitivity of LNCaP to cell death. *PLoS One* 2013; 8(2):e54920

Fritz IB, Burdzy K, Sétchell B, Blaschuk O**.** Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions *in vitro*. *Biol of Reprod* 1983; 28:1173–1182.

Goetz EM, Shankar B, Zou Y, Morales JC, Luo X, Araki S, *et al*. ATM-dependent IGF-1 induction regulates secretory clusterin expression after DNA damage and in genetic instability. *Oncogene* 2011;30:3745–3754.

Han BH, DeMattos RB, Dugan LL, Kim-Han JS, Brendza RP, Fryer JD, *et al*. Clusterin contributes to caspase-3-independent brain injury following neonatal hypoxiaischemia. *Nat Med* 2001; 7(3):338–343.

Hellebrekers DM, Melotte V, Viré E, Langenkamp E, Molema G, Fuks F, *et al*. Identification of epigenetically silenced genes in tumor endothelial cells. *Cancer Res* 2007; 67:4138–4148.

Jin G, Howe PH. Transforming growth factor beta regulates clusterin gene expression via modulation of transcription factor c-Fos. *Eur J Biochem* 1999; 263(2):534–542.

Jo H, Jia Y, Subramanian KK, Hattori H, Luo HR. Cancer Cell-Derived Clusterin modulates the Phosphatidylinositol 3‟-Kinase-Akt pathway through attenuation of Insulin-Like growth factor 1 during serum deprivation. *Mol Cell Biol* 2008; 28(13):4285–4299.

Jones S, Jomary C. Molecules in focus: Clusterin. *Int J Biochem Cell Biol* 2002;34(5):427–431.

Klokov D, Criswell T, Leskov KS, Araki S, Mayo L, Boothman DA. IR-inducible clusterin gene expression: a protein with potential roles in ionizing radiation-induced adaptive responses, genomic instability, and bystander effects. *Mutation Research* 2004;568: 97–110.

Koltai T. Clusterin: a key player in cancer chemoresistance and its inhibition. *Onco Targets Ther* 2014;7:447–456.

Kong D, Liu S, Wang Q, Jia J, Li N, Zhang K, Jiao X. Targeted knockdown of Clusterin sensitizes pancreatic cancer MIA-PaCa-2 cell to Gemcitabine treatment by inactivation of NF-κB/ Bcl2. *Biomedical Research* 2012;23:SI 91–98.

Lee DH, Ha JH, Kim Y, Bae KH, Park JY, Choi WS, *et al*. Interaction of a putative BH3 domain of clusterin with anti-apoptotic Bcl-2 family proteins as revealed by NMR spectroscopy. *Biochem Biophys Res Commun* 2011; 408:541–547.

Lee KB, Jeon JH, Choi I, Kwon OY, Yu K, You KH. Clusterin, a novel modulator of TGF-beta signaling is involved in Smad2/3 stability. *Biochem Biophys Res Commun* 2008; 366:905–909.

Lee S, Hong SW, Min BH, Shim YJ, Lee KU, Lee IK, *et al*. Essential role of clusterin in pancreas regeneration. *Dev Dyn* 2011; 240(3):605–615.

Lee J, Kim H, Rho S, & Lee S. eIF3f reduces tumor growth by directly interrupting clusterin with anti-apoptotic property in cancer cells. *Oncotarget* 2016;*7*(14),18541–18557.

Léger JG, Montpetit ML, Tenniswood MP. Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. *Biochem Biophys Res Commun* 1987; 147: 196–203.

Leskov K, Klokov D, Li J, Kinsella T, Boothman D. Synthesis and functional analyses of nuclear clusterin, a cell death protein. J Biol Chem 2003; 278: 11590–600.

Li N, Zoubeidi A, Beraldi E, Gleave ME. GRP78 regulates clusterin stability, retrotranslocation and mitochondrial localization under ER stress in prostate cancer. *Oncogene* 2013; 11;32(15):1933–1942.

Li Y, Lu J, Zhou S, Wang W, Tan G, Zhang Z, *et al.* Clusterin induced by N,N′-Dinitrosopiperazine is involved in nasopharyngeal carcinoma metastasis. *Oncotarget* 2016; *7*(5), 5548–5563.

Liao FT, Lee YJ, Ko JL, Tsai CC, Tseng CJ, Sheu GT. Hepatitis delta virus epigenetically enhances clusterin expression via histone acetylation in human hepatocellular carcinoma cells. *J. Gen. Virol* 2009;90:1124–1134.

Ling I, Bhongsatiern J, Simpson JF, Fardo DW, Estus S. Genetics of Clusterin Isoform Expression and Alzheimer's Disease Risk. *PLoS One* 2012;7(4): e33923.

Liu B, Han MT, Zhang J, Lu P, Li J, Song N, *et al.* Downregulation of Clusterin expression in human testicular seminoma. *Cell Physiol Biochem* 2013; 32:1117–1123.

Lund P, Weisshaupt K, Mikeska T, Jammas D, Chen X, Kuban R *et al.* Oncogenic *HRAS* suppresses *clusterin* expression through promoter hypermethylation. *Oncogene* 2006;25:4890–4903.

Luo X, Suzuki M, Ghandhi SA, Amundson SA, Boothman DA. ATM regulates Insulin-Like Growth Factor 1-secretory Clusterin (IGF-1-sCLU) expression that protects cells against senescence. *PLoS One* 2014;9(6): e99983.

McLaughlin L, Zhu G, Mistry M, Ley-Ebert C, Stuart WD, Florio CJ, *et al*. Apolipoprotein J/clusterin limits the severity of murine

autoimmune myocarditis. *J Clin Invest* 2000;106(9):1105–1113.

Miyake H, Gleave ME, Arakawa S, Kamidono S, Hara I. Introducing the Clusterin gene into human renal cell carcinoma cells enhances their metastatic potential. *The Journal of Urology* 2002a; 167(5):2203–2208.

Miyake H, Gleave M, Kamidono S, Hara I. Overexpression of clusterin in transitional cell carcinoma of the bladder is related to disease progression and recurrence. J *Urology* 2002b 59:150–154.

Mydlarz W, Uemura M, Ahn S, Hennessey P, Chang S, Demokan S, *et al.* Clusterin is a gene specific target of microRNA-21 in head and neck quamous cell carcinoma. *Clin Cancer Res* 2014; 20(4):868–877.

Nuutinen T, Suuronen T, Kyrylenko S, Huuskonen J, Salminen A. Induction of Clusterin/apoJ expression by histone deacetylase inhibitors in neural cells. *Neurochem Int* 2005; 47(8): 528–538.

O'Sullivan J, Whyte L, Drake J, Tenniswood M. Alterations in the post-translational modification and intracellular trafficking of clusterin in MCF-7 cells during apoptosis. *Cell Death Differ* 2003;10: 914–27.

Park P, Park S, Shin E, Lee S, Kim Y, Lee D *et al.* Hypoxia inducible factor-1α directly regulates nuclear clusterin transcription by interacting with hypoxia response elements in the clusterin promoter. *Mol. Cells* 2014; 37(2):178–186.

Prochnow H, Gollan R, Rohne P, Hassemer M, Koch-Brandt C, Baiersdörfer M. Non-Secreted Clusterin isoforms are translated in rare amounts from distinct human mRNA variants and do not affect Bax-mediated apoptosis or the NF-κB signaling Pathway. *PLoS One* 2013; 8(9):e75303.

Rauhala HE, Porkka KP, Saramäki OR, Tammela TL, Visakorpi T. Clusterin is epigenetically regulated in prostate cancer. *Int. J. Cancer* 2008;123:1601–1609.

Reddy KB, Jin G, Karode MC, Harmony JA, Howe PH. Transforming growth factor beta induced nuclear localization of apolipoprotein J/clusterin in epithelial cells. *Biochemistry* 1996**;** 35(19):6157–6163.

Rizzi F, Bettuzzi S. The clusterin paradigm in prostate and breast carcinogenesis. *Endocr Relat Cancer* 2010;17: R1–17.

Rohne P, Prochnow H, Wolf S, Renner B, Koch-Brandt C. The chaperone activity of clusterin is dependent on glycosylation and redox environment. *Cell Physiol Biochem* 2014;34:1626–1639.

Rohne P, Prochnow H, Wolf S, Koch-Brandt C. The CLU-files: disentanglement of a mystery. *BioMol Concepts* 2016;7:1–15

Rosenberg ME, Silkensen J. Clusterin: Physiologic and pathophysiologic considerations. *Int J Biochem Cell Biol* 1995; 27:633–645.

Rosenberg ME, Girton R, Finkel D, Chmielewski D, Barrie A, Witte DP,*et al*. Apolipoprotein J/clusterin prevents progressive glomerulopathy of aging. *Mol Cell Biol* 2002;22:1893–1902.

Sala A, Bettuzzi S, Pucci S, Chayka O, Dews M, Thomas-Tikhonenko A. Regulation of CLU gene expression by oncogenes and epigenetic factors: Implications for tumorigenesis. *Advances in cancer research*. 2009;105:115–132.

Santilli G, Aronow BJ, Sala A. Essential

requirement of Apolipoprotein J (Clusterin) signaling for I-κB Expression and Regulation of NF-ƙB Activity. *J Bio Chem* 2003; 278:38214–38219.

Santilli G, Schwab R, Watson R, Ebert C, Aronow BJ, Sala A. Temperature-dependent modification and activation of B-MYB: implications for cell survival. *J Biological Chem* 2005;280:15628–15634.

Schepeler T, Mansilla F, Christensen LL, Orntoft TF, Andersen CL. Clusterin expression can be modulated by changes in TCF1-mediated Wnt signaling. *J Mol Signal* 2007; 2:6.

Sensibar JA, Sutkowski DM, Raffo A, Buttyan R, Griswold MD, Sylvester SR, *et al*. Prevention of cell death induced by tumor necrosis factor α in LNCaP cells by overexpression of sulfated glycoprotein-2 (Clusterin). *Cancer Res* 1995; *55;* 2431–2437.

Serrano A, Redondo M, Tellez T, Castro-Vega I, Roldan M, Mendez R, *et al.* Regulation of clusterin expression in human cancer *via* DNA methylation. *Tumour. Biol* 2009;30:286–291.

Shiiba M, Uzawa K, Tanzawa H. MicroRNAs in Head and Neck Squamous Cell Carcinoma (HNSCC) and Oral Squamous Cell Carcinoma (OSCC). *Cancer* 2010;2(2):653–669.

Shannan B, Seifert M, Leskov K, Willis J, Boothman D, Tilgen W, Reichrath J. Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. *Cell Death Differ* 2006; 13(1):12–19.

Shim YJ, Shin YJ, Jeong SY, Kang SW, Kim BM, Park IS, Min BH. Epidermal growth factor receptor is involved in clusterin-induced astrocyte proliferation. *Neuroreport* 2009; 20(4):435–439.

Shim YJ, Kang BH, Jeon HS, Park IS, Lee KU, Lee IK, *et al*. Clusterin induces matrix metalloproteinase-9 expression via ERK1/2 and PI3K/Akt/NF-ƙB pathways in monocytes/macrophages. *J Leukoc Biol* 2011; 90:761–769.

Shiota M, Zardan A, Takeuchi A, Kumano M, Beraldi E, Naito S, *et al*. Clusterin mediates TGF-β-induced epithelial-mesenchymal transition and metastasis via Twist1 in prostate cancer cells. *Cancer Res* 2012; 72(20):5261–5272.

So A, Rocchi P, Gleave M. Antisense oligonucleotide therapy in the management of bladder cancer. *Curr Opin Urol* 2005; 15: 320–327.

Takeuchi A, Shiota M, Beraldi E, Thaper D, Takahara K, Ibuki N, Pollak M, Cox ME , Naito S, Gleave ME, Zoubeidi A. Insulin-like growth factor-I induces CLU expression through Twist1 to promote prostate cancer growth. *Mol Cell Endocrinol* 2014; 384(1–2):117–125.

Tang Y, Liu F, Zheng C, Sun S, Jiang Y. Knockdown of clusterin sensitizes pancreatic cancer cells to gemcitabine chemotherapy by ERK1/2 inactivation. *Journal of Experimental & Clinical Cancer Research* 2012;31:73.

Thomas-Tikhonenko A, Viard-Leveugle I, Dews M, Wehrli P, Sevignani C, Yu D, *et al.* Myc transformed epithelial cells down-regulate Clusterin, which inhibits their growth *in vitro* and carcinogenesis *in vivo*. *Cancer Res* 2004; 64:3126–3136.

Tremblay G, Viau E, Filion M. Co-use of a

clusterin inhibitor with an egfr inhibitor to treat cancer. WO2013123588 A1.

Trougakos I , Gonos E. Clusterin/apolipoprotein J in human aging and cancer. *Int J Biochem Cell Biol* 2002;34:1430–1448.

Trougakos I, Lourda M, Antonelou M, Kletsas D, Gorgoulis V, Papassideri I, *et al*. Intracellular Clusterin inhibits mitochondrial apoptosis by suppressing p53-activating stress signals and stabilizing the cytosolic Ku70-Bax protein complex. *Clin Cancer Res* 2009;15:48–59.

Trougakos I. The molecular chaperone apolipoprotein J/clusterin as a sensor of oxidative stress: implications in therapeutic approaches – a mini-review. *Gerontology* 2013;59(6):514–523.

Wang Q, Cao W, Su Q, Liu Z, Zhang L. Clusterin silencing inhibits proliferation and reduces invasion in human laryngeal squamous carcinoma cells. *World Journal of Surgical Oncology* 2014;12:124.

Wilson M, Easterbrook-Smith S. Clusterin is a secreted mammalian chaperone. *Trends Biochem Sci* 2000**;**25(3):95–98.

Wong P, Taillefer D, Lakins J, Pineault J, Chader G, Tenniswood M. Molecular characterization of human TRPM-2/clusterin, a gene associated with sperm maturation, apoptosis and neurodegeneration. *Eur J. Biochem* 1994; 221: 917–925.

Wyatt AR, Yerbury JJ, Wilson MR. Structural characterization of Clusterin-chaperone client protein complexes. *J Biol Chem*. 2009; 284:21920–21927.

Xie MJ, Motoo Y, Su SB, Mouri H, Ohtsubo K, Matsubara F, *et al.* Expression of clusterin in human pancreatic cancer. *Pancreas* 2002; 25(3):234–238.

Zellweger T, Miyake H, Cooper S, Chi K, Conklin BS, Monia BP, Gleave ME. Antitumor activity of antisense clusterin oligonucleotides is improved *in vitro* and *in vivo* by incorporation of 2′-o- (2-methoxy) ethyl chemistry. *J Pharmacol Exp Ther* 2001;298: 934–940.

Zhang L, Ying W, Mao Y, He H, Liu Y, Wang H, *et al.* Loss of clusterin both in serum and tissue correlates with the tumorigenesis of esophageal squamous cell carcinoma via proteomics approaches. *World Journal of Gastroenterology*, vol. 9, no. 4, pp. 650–654, 2003.

Zhang H, Kim JK, Edwards CA, Xu Z, Taichman R, Wang CY. Clusterin inhibits apoptosis by interacting with activated Bax. *Nat Cell Biol* 2005;7(9): 909–915.

Zhang B, Zhang K, Liu Z, Hao F, Wang M, Li X, Yin Z, Liang H. Secreted Clusterin gene silencing enhances chemosensitivity of A549 cells to cisplatin through AKT and ERK1/2 pathways *in vitro. Cell Physiol Biochem* 2014; 33:1162–1175.

Zhang F, Kumano M, Beraldi E, Fazli L, Du C, Moore S, Sorensen P, Zoubeidi A, Gleave ME. Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival. *Nat Commun* 2014;5:5775.

Zoubeidi A, Ettinger S, Beraldi E, Hadaschik B, Zardan A, Klomp LW, Nelson CC, *et al*. Clusterin facilitates COMMD1 and I-κB degradation to enhance NF-κB activity in Prostate cancer cells. *Mol Cancer Res* 2010; 8:119–130.

Review

BRJ
Biomedical Research Journal

Chemoprotectants in Cancer Chemotherapy: An Update

Abhishek Basu, Arin Bhattacharjee, and Sudin Bhattacharya*

Department of Cancer Chemoprevention, Chittaranjan National Cancer Institute, Kolkata – 700026, West Bengal, India

Cancer chemotherapeutic agents play an integral part in the management of patients with malignancy. However, chemotherapy is associated with significant toxicity with an adverse impact on the health of the patients. As a result the therapeutic outcome is influenced due to the inability to deliver sufficient dose-intensive therapy leading to treatment delays or cessation. Chemoprotectants have been developed in order to mitigate the toxicity associated with chemotherapeutic agents by providing organ-specific protection to normal tissues, without compromising the antitumor efficacy. The current review highlights chemoprotectants in the management of chemotherapeutics-associated toxicity, such as: amifostine, aprepitant, dexrazoxane, filgrastim, sargramostim, mesna, oprelvekin, palifermin, recombinant human erythropoietin etc. Additionally, the present status on the concurrent use of chemoprotectants in combination with chemotherapeutic agents, with focus on their safety is included. The advantageous role of these cytoprotective agents combined with chemotherapy remains controversial in clinical studies due to moderate protective efficacy for normal tissues and organs, risk of concomitant tumor protection and adverse reactions. Besides, the number of successful agents is rather small. Therefore, identification of novel approaches and chemoprotectants holds potential for better management of cancer with chemotherapy.

INTRODUCTION

Cytotoxic antineoplastic agents play integral part in the management of cancer patients. However, the chemotherapeutic agents are cytotoxic to the malignant cells, and also affect normal cells (DeVita and Chu, 2008). This results in a narrow therapeutic index coupled with severe form of toxicity impacting adversely on the quality of the life of the patients. Furthermore, the adverse effects result in treatment delays, sub-therapeutic dose delivery and cessation of treatment, and impact the treatment outcome and patient survival (Braun and Seymour, 2011). A summary of common form of chemotherapy-induced toxicities is demonstrated in Table 1.

A better understanding of the cancer

Key words: Chemoprotection, cytoprotective agents, chemotherapy, nutraceuticals, antioxidants, growth factors.
***Corresponding Author:** Sudin Bhattacharya, Department of Cancer Chemoprevention, Chittaranjan National Cancer Institute, 37, S. P. Mukherjee Road, Kolkata – 700 026, West Bengal, India.
Email: sudinb19572004@yahoo.co.in

Table 1: Common form of chemotherapy-induced toxicity.

Types of toxicity	Chemotherapeutic agents		
	Severely toxic	**Moderately toxic**	**Mildly toxic**
Myelotoxicity	Alkylating agents, Anthracyclins, Carboplatin, Cytarabine, Etoposide, Taxanes	Cisplatin, Fluorouracil, Ifosfamide, Methotrexate	Vinca alkaloids
Gastrointestinal toxicity	Anthracyclins	Cytarabine, Etoposide, Fluorouracil, Methotrexate, Nitrosoureas	Alkylating agents, Bleomycin, Cisplatin, Carboplatin, Ifosfamide, Taxanes
Hepatotoxicity		Anthracyclins, Nitrosoureas	Cytarabine, Fluorouracil, Methotrexate, Taxanes
Nephrotoxicity	Cisplatin	Ifosfamide, Methotrexate, Nitrosoureas	Carboplatin
Pulmonary toxicity	Bleomycin	Nitrosoureas	Alkylating agents, Fluorouracil, Ifosfamide, Methotrexate
Peripheral nephropathy	Cisplatin, Taxanes, Vinca alkaloids		Carboplatin
CNS toxicity		Ifosfamide	
Cardiac toxicity	Anthracyclins		Alkylating agents, Fluorouracil, Ifosfamide, Taxanes
Hemorrhagic cystitis	Cyclophosphamide, Ifosfamide		Alkylating agents
Alopecia	Anthracyclins, Etoposide, Taxanes	Alkylating agents, Ifosfamide	Bleomycin, Cytarabine, Nitrosoureas

cell biology was anticipated to identify specific targets for cancer therapy. However, a need for strategies to reduce or circumvent host organ toxicity is the need of the hour (Liu *et al*., 2015). The chemoprotective therapies have been developed to mitigate the healthy tissue toxicity and improve the therapeutic window of cytotoxic antineoplastic agents. Chemoprotection is defined as protection of the toxicity of a chemical through administration of another agent (Jena *et al*., 2010). An ideal chemoprotectant should be easy to administer, non-toxic, not alter the pharmacokinetics of the cytotoxic agent

and should not inhibit or reduce antitumor activity of the drug (Marx and Friedlander, 2010). To cite an example, reactive oxygen species (ROS) generated by anticancer drug or a free radical intermediate of the drug plays a critical role in cytotoxicity of cancer cells, then antioxidative chemoprotectant is not indicated as it will interfere with the antineoplastic activity. However, if generation of ROS is responsible only for the adverse effects of the anticancer drug, then antioxidative chemoprotectant may reduce the severity of the toxicity without interfering with the antineoplastic activity of the drug (Conklin, 2004). The first chemoprotectant in clinical use was folinic acid (calcium folinate; leucovorin), indicated to circumvent methotrexate-induced toxicity (Links and Lewis, 1999).

During chemotherapy, selection of chemotherapeutic agents, and the dose and duration of treatment is dependent on the type and stage of malignancy. However, consideration to selection of appropriate chemoprotectants is often neglected and is equally important (Jena *et al*., 2010). The efficacy of various chemoprotectants differs in terms of potency, pharmacokinetics, accumulation, distribution, and mechanism of action; and hence, these parameters must be taken into account during selection of chemoprotectants for clinical use. It is difficult and perhaps impossible to design a common chemoprotectant to circumvent the deleterious effects, irrespective of individual therapy (chemo or radiation). Thus, the complexity still lies in appropriate selection of chemoprotectants and their use in chemotherapy or radiotherapy without compromising the efficacy. In the current review, currently used chemoprotective agents, their clinical use and limitations have been highlighted.

Amifostine (Ethyol®)

Amifostine (WR-2721, S-2-[3-aminopropylamino] ethylphosphoro-thioic acid) (Fig.1) is a prodrug converted to the active, dephosphorylated, cell permeable metabolite WR-1065 by cell membrane-bound alkaline phosphatase (Hoekman *et al*., 1999), initially used for capability to prevent damage caused by ionizing radiation (Kouvaris *et al*., 2007). It is a broad-spectrum cytoprotectant specific for host organs and tissues and

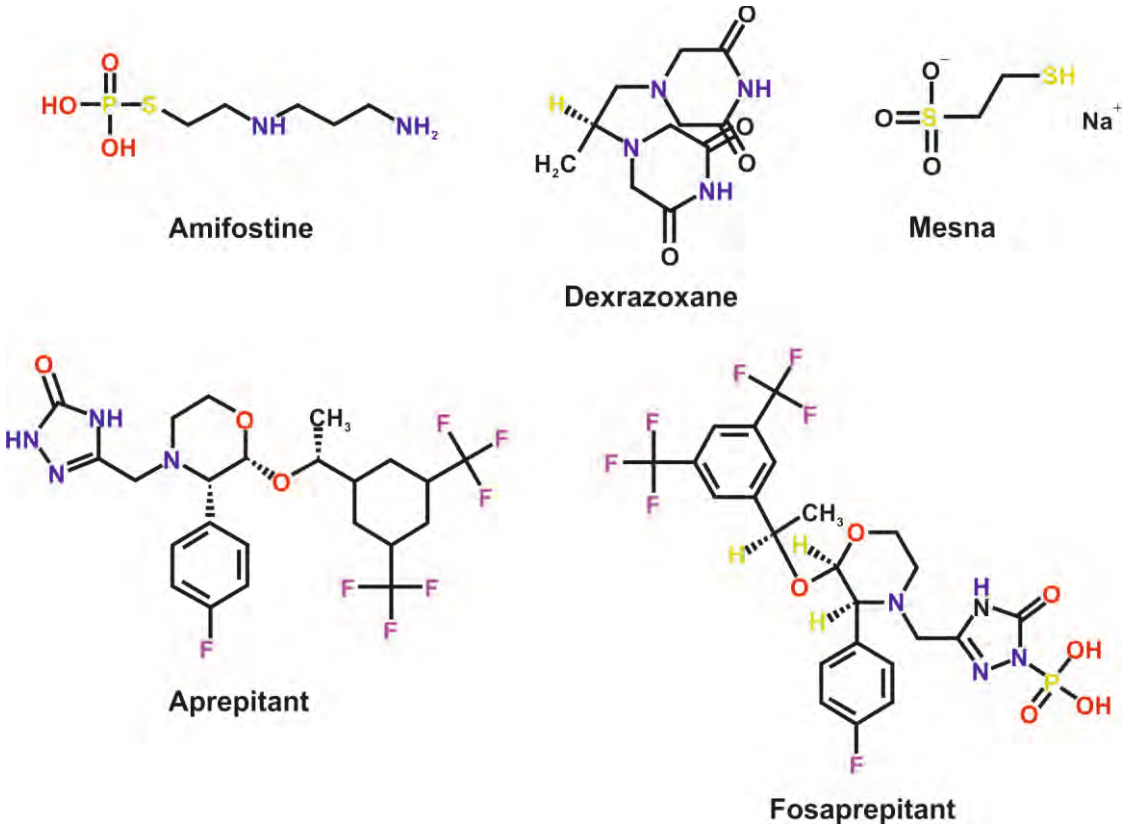


Figure 1: Chemical structure of some clinically used chemoprotectants.

recommended by US Food and Drug Administration (USFDA) for clinical use in patients receiving cisplatin alone and/or in combination with other chemotherapeutic drugs (Ali and Al Moundhri, 2006; Devine and Marignol, 2016). The American Society of Clinical Oncology endorsed amifostine use in prevention of cisplatin-associated nephrotoxicity, for minimization of neutropenia (grade 3–4), and reduce acute and late xerostomia associated with radiotherapy in head and neck cancer (Nicolatou-Galitis *et al*., 2013).

The metabolite of amifostine, WR-1065 is suggested to be responsible for the chemoprotective efficacy of amifostine. Amifostine selectively protects normal organs and tissues due to the greater capillary alkaline phosphatase activity, high pH and superior vascularity of normal tissues in comparison to tumor tissue (van den Berg *et al*., 2006). Thus, normal calls may be able to acquire about 100-fold higher concentration of the free thiol than tumor cells (Marx and Friedlander, 2010). Intracellularly, WR-1065 scavenges free radicals, protecting DNA and cellular membranes from damage (Kouvaris *et al*., 2007). The

oxidation of WR-1065 to WR-33278 (polyamine-like disulfide metabolite) results in higher amount of WR-33278 conjugated DNA, thereby restricting target sites against free radical attack (Savoye *et al*., 1997). Thus WR-1065 contributes to minimization of double-strand breaks following chemotherapy, resulting in recovery of the temporary block of cell cycle at G_2 phase, thereby promoting proliferation of epithelial cells (Rubin *et al*., 1996). Indirectly, amifostine through induction of hypoxia stimulates expression of proteins implicated in DNA repair and inhibition of apoptosis, such as HIF-1α and Bcl-2 (Kouvaris *et al*., 2007).

Amifostine exerts protection as reported in several clinical trials against cisplatin-induced nephrotoxicity and cyclophosphamide-induced hemato-toxicity (Links and Lewis, 1999). The recommended dose for amifostine is 740–910 mg/m^2. Amifostine is well tolerated with the main toxicities being nausea, sneezing, allergic reactions, metallic taste and hypotension. Transient hypocalcaemia has been also noted and is due to the deregulation of parathyroid hormone (Marx and Friedlander, 2010). Clinical trials in advanced ovarian cancer patients confirmed that pre-treatment with amifostine effectively attenuate the cumulative renal, hematologic and neurologic toxicity of the chemotherapy regimen constituting cisplatin and cyclophosphamide (Devine and Marignol, 2016; Kemp *et al*., 1996). Different amifostine analogues have been investigated preclinically to define toxicity. Amongst these, DRDE-07 (S-2 (2-aminoethylamino) ethyl phenyl sulfide) showed most promising efficacy (Gautam *et al*., 2010).

Aprepitant (Emend®)

Chemotherapy-induced nausea and vomiting (CINV) are adverse effects on the quality of life of patients (Ballatori and Roila, 2003). The incidence of CINV influences patient compliance with chemotherapeutic regimens, and influences the decision of patient to undergo chemotherapeutic treatment (Aapro *et al*., 2015). Aprepitant (Fig.1) has emerged as a new class of antiemetic for control of CINV (Grunberg *et al*., 2013). Recent clinical regulations from the Multinational Association for Supportive Care in Cancer (MASCC), European Society of Medical Oncology (ESMO), American Society of Clinical Oncology (ASCO), and the National Comprehensive Cancer Network

(NCCN) approved aprepitant singly or in combination with serotonin receptor antagonist or corticosteroid, as the most effective therapeutic regimen for reducing both acute and delayed CINV associated with high emetic chemotherapy, or with anthracycline, cyclophosphamide and/or cisplatin-based therapeutic regimens (Aapro *et al*., 2015; Basch *et al*., 2011).

Aprepitant is a highly selective antagonist of human substance P or neurokinin 1 (NK1) receptors. Aprepitant has little or no affinity for dopamine, serotonin (5-HT_3), and corticosteroid receptors, the molecular targets of existing therapies for CINV and postoperative nausea and vomiting (PONV) (Hargreaves *et al*., 2011). Animal and human studies with aprepitant have revealed that by crossing the blood brain barrier it occupies brain NK1 receptors (Bergström *et al*., 2004). Aprepitant augments the antiemetic activity of dexamethasone and 5-HT_3 receptor antagonist ondansetron, and blocks the acute and delayed phases of emesis induced by cisplatin (Di Maio *et al*., 2013). The usual toxicity associated with aprepitant is constipation, tiredness, headache, loss of appetite, and hair loss. In some cases, incidence of pruritus and neutropenia are reported (Aapro *et al*., 2013).

Fosaprepitant (Ivemend®) (Fig.1) is a newly marketed intravenous prodrug formulation of aprepitant. USFDA and European Medicines Agency (EMEA) approved fosaprepitant for prevention of acute and delayed nausea and vomiting associated with initial and repeated courses of moderate to high emetogenic cancer chemotherapy, including high-dose cisplatin (Langford and Chrisp, 2010). Several other NK1 receptor antagonists including casopitant, rolapitant, and netupitant, are undergoing clinical studies for management of CINV (Aapro *et al*., 2015). Casopitant had completed numerous phase III trials, but was not approved by the USFDA because of insufficient safety data (Navari, 2013). Both netupitant and rolapitant were promising in control of CINV. Rolapitant is under phase III trials. Netupitant in combination with palonosetron showed efficiency in reducing CINV in phase III trials (Aapro *et al*., 2014).

Dexrazoxane (Zinecard®)

Dexrazoxane (ICRF-187), a bisdiozpiperazine (Fig.1), is the *d*-isomer of the racemic compound razoxane (ICRF-159) and a lipophilic derivative of

ethylenediaminetetraacetic acid (EDTA), a chelating agent (Hoekman *et al*., 1999). Dexrazoxane has received USFDA approval to minimize the incidence and severity of doxorubicin-associated cardiomayopathy in women with metastatic breast cancer. In UK Dexrazoxane is used for prevention of doxorubicin- or epirubicin-induced chronic cumulative cardiotoxicity in advanced/metastatic cancer patients following anthracycline-therapy (Jones, 2008).

The cardioprotective activity is due to the hydrolysis product ICRF-198 (hydrolyzed by dihydropyrimidine aminohydrolase), which chelates the free and bound forms of myocardial intracellular iron, subsequently decreasing complexation of metal ions with anthracycline, hence leading to a decline in the formation of superoxide anions (Jones, 2008). In addition, dexrazoxane also shows cytotoxic effect via inhibition of topoisomerase II (Zhang *et al*., 2012), and thus potentiates or antagonizes the cytotoxicity of chemotherapeutic agents in experimental tumor models (Hasinoff *et al*., 1998; Sehested *et al*., 1993). Dexrazoxane diminishes doxorubicin-induced cardiotoxicity through its capability to inhibit topoisomerase IIβ (Zhang *et al*., 2012), and degrades topoisomerase IIβ, reducing doxorubicin-induced DNA damage (Lyu *et al*., 2007).

Randomized clinical trials have established the chemoprotective efficacy of dexrazoxane against anthracycline-induced cardiac damage (Doroshow, 2012). Besides, dexrazoxane potentiates hematotoxicity caused by chemotherapy or radiation (Links and Lewis, 1999). The common adverse effects are phlebitis at the site of injection and myelotoxicity (Hoekman *et al*., 1999). Dexrazoxane has been associated with a greater risk of developing secondary malignancy, such as, acute myeloid leukemia and myelodysplastic syndrome in pediatric patients with Hodgkin's disease (Jones, 2008). Recently, dexrazoxane was used as an antidote for anthracycline-induced extravasation injury (Doroshow, 2012).

Filgrastim (Neupogen®) and Sargramostim (Leukine®)

The hematopoietic growth factors (HGFs) are a family of endogenous glycoproteins with a role in survival, proliferation, and differentiation of primordial hematopoietic progenitor and stem cells, and regulation of certain adult cells (Raposo *et al*., 2006). Twenty

molecules of HGF have been characterized, with granulocyte colony-stimulating factor (filgrastim) and granulocyte-macrophage colony-stimulating factor (sargramostim) indicated for reducing febrile neutropenia following chemotherapy and as a supportive therapy in bone marrow transplantation (Mhaskar *et al*., 2014). Filgrastim and sargramostim have been approved for therapy by USFDA on 1991 (Beveridge *et al*., 1998).

Filgrastim is an analog of granulocyte colony-stimulating factor (G-CSF) biosynthesized in *Escherichia coli* by recombinant DNA technology (Sourgens and Lefrère, 2011). Filgrastim stimulates production of neutrophils in the bone marrow, induces proliferation and differentiation of neutrophil progenitor cells, enhances phagocytic ability, antibody dependent killing, priming of the cellular metabolism associated with respiratory burst, and enhances expression of certain cell surface antigens (Haas and Murea, 1995). On the other hand, sargramostim is a yeast-derived recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Waller, 2007). During hematopoiesis, sargramostim induces growth of macrophage, granulocyte, lymphocytes and eosinophil colonies (Raposo *et al*., 2006). It generates myeloid dendritic cells and monocytes, leading to greater immunogenic responses, against tumor specific antigens (Waller, 2007). Sargramostim acts on tumor cells by cytokine priming (Boyer *et al*., 2000). In acute myelogenous leukemia (AML), Sargramostim enhances the susceptibility of leukemic blast cells to antitumor activity of chemotherapy. It causes terminal differentiation of cancer stem cells to myeloid cells, thus reducing the number of self-renewing cells (Arellano *et al*., 2007), differentiates the blasts to antigen-presenting cells that activate immune responses and targets the cells for immunotherapy (Boyer *et al*., 2000).

Filgrastim and sargramostim are administered as a prophylactic or curative therapy in patients on myeloablative chemotherapy resulting in prolonged neutropenia. Patients with AML, Hodgkin's lymphoma, non-Hodgkin's lymphoma, sarcomas, seminomas and small cell carcinomas of the lungs are treated with these agents (Raposo *et al*., 2006). Before collection by leukapheresis for hematopoietic stem cell transplantation, Filgrastim is used to augment hematopoietic stem cells in

blood (Kelsey *et al*., 2016). Sargramostim is also indicated in neutropenic patients with myelodysplastic syndrome (MDS) and/or aplastic anemia (Mehta *et al*., 2015). Therapy is usually begun 24–72 hours after cessation of chemotherapy and is often continued until the absolute neutrophil count reaches a normal count of 10,000 cells/µl (Mehta *et al*., 2015). The major associated toxicity includes flu-like symptoms of flushing, rash, fever, malaise, arthralgia, myalgia, headache, anorexia and elevations of serum aminotransferases (Henk *et al*., 2015).

Mesna (Mesnex®)

Mesna (sodium-2-mercapto-ethane sulfonate) (Fig.1) is a specific chemoprotectant against hemorrhagic cystitis induced by cyclophosphamide and ifosfamide (Altayli *et al*., 2012). Cyclophosphamide and ifosfamide undergo biotransformation by hepatic microsomal enzymes to form acrolein and phosphoramide mustard. Acrolein and related urotoxic metabolites, especially 4-hydroxy metabolites (4-hydroxy-ifosfamide and 4-hydroxy-cyclophosphamide) are consequently excreted into the urinary bladder to induce hemorrhagic cystitis (Zhang *et al*., 2006). The incidence of hemorrhagic cystitis following high-dose cyclophosphamide ranges from 0.5-40% in patients (Marx and Friedlander, 2010). Being a thiol compound mesna inactivates alkylating metabolites forming an inert form of thioether. In the bloodstream, mesna is converted to an inactive disulfide form, dithiodiethanesulfate or dimesna. Dimesna is subsequently secreted and filtered in the kidneys, where the enzymes glutathione reductase and thiol transferase reducing dimesna to mesna. Mesna then enters in the bladder, where the free sulfhydryl groups forms a conjugate with acrolein (Links and Lewis, 1999). Mesna also binds to 4-hydroxy-ifosfamide or 4-hydroxy-cyclophosphamide to form a non-urotoxic 4-sulfoethylthio-ifosfamide or 4-sulfoethylthio-cyclophosphamide (Salman *et al*., 2016). As the efficacy of mesna is limited to urinary tract, the non-urological toxicity and the systemic activity of the oxazaphosphorines are not affected. Hence combinatorial treatment with mesna and cyclophosphamide or ifosfamide is effective (Links and Lewis, 1999).

Several clinical studies have

confirmed efficacy of mesna against cyclophosphamide- and ifosfamide-induced bladder toxicity (Salman *et al*., 2016). However, 5% of patients on mesna and cyclophosphamide or ifosfamide therapy suffer from hemorrhagic cystitis during or on completion of the treatment. This may be due to additional metabolites such as chloroethylaziridine and phosphoramide mustard including hemorrhagic cystitis and mesna does not inactivate the agents that cause symptoms of hemorrhagic cystitis (Altayli *et al*., 2012). Mesna minimizes hematuria and hemorrhagic cystitis in patients receiving cyclophosphamide or ifosfamide during chemotherapy (Payne *et al*., 2013). Mesna is also indicated as a mucolytic agent (Sathe *et al*., 2015).

Mesna is generally administered intravenously or orally, with 2 litre of intravenous or oral fluid daily for ensuring hydration. Therapeutic cycles are generally repeated every 3-4 weeks (Links and Lewis, 1999). Mesna is usually associated with minimal toxicity. The most frequently reported adverse effects were headache, dizziness, nausea, vomiting, diarrheal, anorexia, back pain, arthralgia, hyperaesthesia, influenza-like symptoms and coughing (Khaw *et al*., 2007).

Oprelvekin (Neumega®)

Interleukin eleven (IL-11) is a thrombopoietic growth factor that activates proliferation and differentiation of hematopoietic stem cells and megakaryocyte progenitor cells, and induces maturation of megakaryocyte leading to enhanced production of platelet (Cantor *et al*., 2003). Interleukin-11 mRNA extracted from MRC5 human fetal lung fibroblast cell line was used to generate a 178 amino acid encoding cDNA, and biosynthesized in *Escherichia coli*. Oprelvekin is nonglycosylated with a molecular mass of 19kD (Wilde and Faulds, 1998).

Oprelvekin was approved by USFDA for prevention of severe form of thrombocytopenia and in patients with non-myeloid malignancies needing platelet transfusions following myeloablative chemotherapy in patients (Sitaraman and Gewirtz, 2001). Thus it was a pharmacological alternative to platelet transfusions, inducing megakaryocytopoiesis and thrombopoiesis (Adams and Brenner, 1999). The induced platelets are

morphologically and functionally normal with normal life span (Berl and Schwertschlag, 2000). The drug is under investigation for management of inflammatory disorders including rheumatoid arthritis, inflammatory bowel disease, and chemotherapy-associated mucositis (Dorner *et al*., 1997). The non-hematopoietic activity of oprelvekin includes inhibition of adipogenesis, regulation of intestinal epithelium growth, stimulation of osteoclastogenesis and neurogenesis, and inhibition of proinflammatory cytokine production by macrophages (Du and Williams, 1997). However, non-hemopoietic pathological alterations observed in animals include periosteal thickening, fibrosis of tendons and joint capsules, papilledema and embryotoxicity (Smith JW, 2001).

The drug is given subcutaneously, injected in the abdomen, hip or thigh post completion of chemotherapy. Administration must be continued until the platelet count is ≥ 50,000 cells/µl; although administration for more than 21 days is not recommended. Oprelvekin must be discontinued at least 2 days before the subsequent cycle of chemotherapy (Kaye, 1998; Wilde and Faulds, 1998). The drug is not indicated in myelotoxic chemotherapy in pediatric patients as the safety and efficacy have not been established (Cantor *et al*., 2003). The most commonly occurring adverse events are dyspnea, edema, palpitations, tachycardia, pleural effusions, atrial fibrillation/flutter, conjunctivitis and oral moniliasis. Adverse effects include an increase in plasma volume and fluid retention, indicating that oprelvekin should be prescribed with caution in patients with congestive heart failure (Baldo *et al*., 2014).

Palifermin (Kepivance®)

Palifermin is a curtailed derivative of keratinocyte growth factor (KGF or FGF7) produced in *Escherichia coli* using recombinant DNA technology (Finch *et al*., 2013). Palifermin is an aqueous-soluble, 140 amino acid, 16.3 kD protein. The first 23 N-terminal amino acids have been deleted to improve protein stability and thus differ from endogenous human KGF (Baldo *et al*., 2014). Palifermin induces cellular growth responses via FGFR2b receptor, is expressed in oesophagus, buccal mucosa, stomach, salivary gland, intestine, liver, lung, kidney, pancreas, bladder, mammary glands, prostate, lens of the eye, skin and thymus (Vadhan-Raj

et al., 2013). Palifermin shows multiple pharmacological activities such as protection and regeneration of the mucosal epithelium following radiation- and chemotherapy- induced damage. Palifermin causes inhibition of DNA damage and apoptosis of epithelial cells, elevation of detoxifying enzymes and attenuation of pro-inflammatory mediators, along with enhanced proliferation, differentiation and migration of epithelial cells (Blijlevens and Sonis, 2007). Palifermin regulates helper Tcell type1 proinflammatory cytokines and increases helper Tcell type2 antiinflammatory cytokines such as IL4 and IL-13 (Panjwani, 2013).

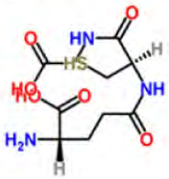
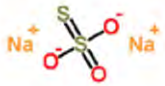
Clinical use of palifermin to minimize the incidence and duration of severe oral mucositis in patients with hematological malignancies undergoing myeloablative therapy has been recommended by USFDA (Chaveli-López and Bagán-Sebastián, 2016). Palifermin mitigates oral mucositis in patients receiving synchronous chemotherapy/radiotherapy or multi-cycle chemotherapy to treat solid tumors. Efficacy in immune reconstitution after hematopoietic stem cell transplantation and decreasing graft-versus-host disease (GVHD) following allogeneic transplantation is under investigation (Vadhan-Raj *et al*., 2013). Intravenous bolus injection is the recommended route of delivery after myelotoxic chemotherapy (Finch *et al*., 2013).

Palifermin is well tolerated, although side effects such as temporary changes in taste, thickening of buccal mucosa and tongue, white coating of tongue, burning sensation and erythema in skin, pruritus, rash and transient elevation in amylase and lipase have been reported (Vadhan-Raj *et al*., 2013). As palifermin acts as a growth factor for epithelial cells and several carcinomas express FGFR2b, it may potentiate tumor growth, block apoptosis and protect tumor cells from chemotherapy (Baldo *et al*., 2014).

Other Chemoprotective Agents

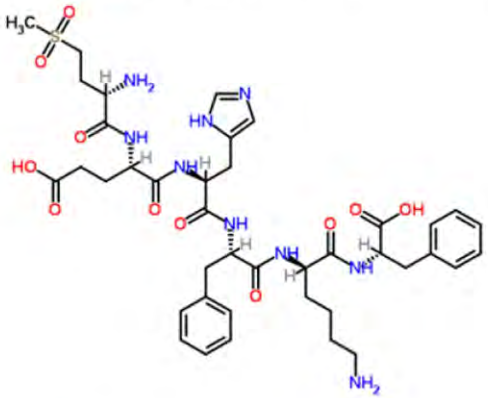
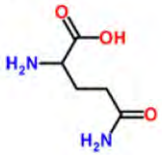
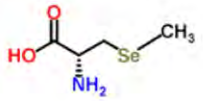
Besides the chemoprotectants mentioned above, potential clinically relevant chemoprotective agents have been indicated in Table 2. These agents act by interfering with the metabolic and cellular regulatory pathways of chemotherapeutics agents, modifications of inflammatory pathways, and antioxidative mechanisms. Herein, the therapeutic indications, mechanism of action and adverse reactions are tabled (Table 2). Apart from the clinically used

Table 2: Clinically relevant chemoprotective agents

Agents	Therapeutic indication	Mechanism of action	Adverse reactions/ Complications	References
Recombinant human erythropoetin (rhEPO)	Chemotherapy-induced anemia	RhEPO, produced by DNA recombinant technology, stimulates production and maturation of red blood cells. RhEPO acts through its transmembrane receptor (EPO-R). The interaction of ligand and receptor causes activation of JAK2 by transphosphorylation, Src signaling, STAT regulation of genes for cell division and differentiation.	Myalgia, iron deficiency, hypertension, seizures and thromboembolism.	Baldo *et al.*, 2014
Glutathione (γ-glutamine-cysteine-glycine) 	Cisplatin-induced neuropathy, renal and systemic toxicity	Exerts cytoprotective effects. Maintains the active form of glutathione peroxidase for scavenging toxic peroxides. Forms intracellular complexes with cisplatin. Glutathione regulates the kinetics of several ion channels, of importance for the biological integrity of the cell.	Elevated level of glutathione in cancer cells confers resistance to chemotherapeutic agents.	Jena *et al.*, 2010; Traverso *et al.*, 2013
Sodium thiosulfate (STS) 	Cisplatin-induced nephrotoxicity, Chemotherapy-induced extravasation injuries	Neutralizes chemotherapeutic agents by converting them into nontoxic species. Does not interact with intracellular concentration of chemotherapeutic agents.	Arthralgia, blurred vision, hyperreflexia, muscle cramps, nausea and vomiting, psychotic behaviour, tinnitus.	Kreidieh *et al.*, 2016

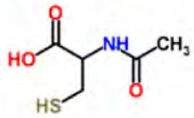
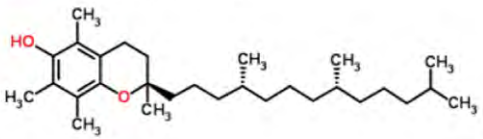
Contd…

Table 2: Clinically relevant chemoprotective agents (Contd…)

Agents	Therapeutic indication	Mechanism of action	Adverse reactions/ Complications	References
ORG-2766 (Analog of corticotropin) 	Neuropathy induced by Paclitaxel, Vincristine, Cisplatin	Hypothesized that ORG-2766 mimics an endogenous peptide, which stimulates the recovery of damaged neurons.	No significant adverse effects reported	Hershman *et al*., 2014
Glutamine 	Methotrexate-, Fluorouracil-induced gastrointestinal toxicity, Cyclophosphamide-induced systemic toxicity, Anthracycline-induced cardiotoxicity, Taxanes-induced neurotoxicity	Protective role through upregulation of GSH, and induces heat shock protein 72 (HSP 72). As a ‘‘stress response’’ induced in response to cell stressors.	Chest pain, nausea, vomiting, abdominal pain, flatulence, arthralgia, depression and edema.	Gaurav *et al*., 2012
5-Methylselenocysteine (MSC) 	Cisplatin-induced hematological, renal and ototoxicity	MSC induces downregulation of reactive oxygen species (ROS) leading to stabilization of prolylhydroxylase (PHD) 2 and 3 with consequent degradation of HIF-1α. MSC down regulates COX2, and iNOS2.	Mild toxicity in liver and kidneys.	Bhattacharya, 2011

Contd…

Table 2: Clinically relevant chemoprotective agents (Contd…)

Agents	Therapeutic indication	Mechanism of action	Adverse reactions/ Complications	References
N-acetyl-L-cysteine (NAC) 	Cyclophosphamide-, Ifosfamide-induced hemorrhagic cystitis, Cisplatin-induced ototoxicity	Serves as a prodrug to L-cysteine and sulfhydryl groups, and causes reduction of extracellular cystine to cysteine. Stimulates glutathione synthesis, enhances glutathione-*S*-transferase activity, promotes liver detoxification by inhibiting xenobiotic biotransformation, and scavenges free radicals. Possesses anti-inflammatory effects possibly via inhibiting NF-κβ and modulating cytokine synthesis.	Rash, urticaria, pruritus, hypotension, wheezing, and shortness of breath. May accelerate tumor growth by disrupting the ROS-p53 axis apoptosis.	Radomska-Leśniewska and Skopiński, 2012
α-Tocopherol (Vitamin E) 	Chemotherapy-induced systemic toxicity, especially peripheral neuropathy	Vitamin E is a peroxyl radical scavenger, disabling production of damaging free radicals in tissues. Treatment with α-tocopherol downregulated expression of CD36 scavenger receptor gene and scavenger receptor class A (SR-A); and modulates expression of the connective tissue growth factor (CTGF). *CTGF* expression results in repair of wounds and regeneration of extracellular tissues damaged during chemotherapy. Protects lipids and prevents oxidation of polyunsaturated fatty acids.	Nausea, diarrhea, stomach cramps, blurred vision, rash, bruising and bleeding. α-tocopherol may increase the possibility of hemorrhagic stroke in brain.	Nakayama *et al.*, 2011

Contd…

Table 2: Clinically relevant chemoprotective agents (Contd…)

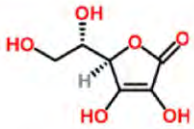
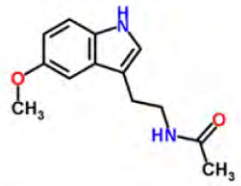
Agents	Therapeutic indication	Mechanism of action	Adverse reactions/ Complications	References
Vitamin C (Ascorbic acid) 	Chemotherapy-related symptoms, such as fatigue, insomnia, loss of appetite, nausea, and pain.	Vitamin C is a potent antioxidant scavenges free radicals and reactive oxygen species. High-dose I.V. vitamin C reduces inflammation, indicated by levels of C-reactive protein (CRP), tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and the interleukins IL-1, IL2, IL-6, IL-8, in cancer patients.	Indigestion, diarrhea and skin rashes. Vitamin C interferes with antitumor activity of methotrexate, dacarbazine and doxorubicin.	Carr *et al*., 2014
Melatonin (*N*-acetyl-5-methoxy tryptamine) 	Chemotherapy-induced systemic toxicity	Melatonin eliminates free radicals, and also induces production of antioxidant enzymes. Melatonin is immunomodulatory and endocrine-modulatory.	Headaches, dizziness, nausea and drowsiness	Seely *et al*., 2012

Table 3: Promising preclinical chemoprotective compounds.

Compounds	Chemoprotective efficacy	Mechanism of action	References
Epigallocatechin-3-gallate (ECGC)	Cyclophosphamide-induced systemic toxicity and DNA damage	Acts as an antioxidant, reduces lipid peroxidation and genotoxicity	Sai Sampath *et al*., 2011
Selenium nanoparticle (Nano-Se)	Cyclophosphamide-induced hepatotoxicity, pulmonary toxicity and genetic damage	Mitigates oxidative stress, DNA damage and enhances antioxidant status	Bhattacharjee *et al*., 2014; Bhattacharjee *et al*., 2015
Indole-3-carbinol (I3C)	Cyclophosphamide-induced developmental toxicity and teratogenicity	Attenuates limb malformation and tail malformation	Bailey et al., 2005
Resveratol	Doxorubicin-induced cardiotoxicity	Ameliorates activity of Na^+,K^+-ATPase and antioxidant enzymes	Tatlidede *et al*., 2009
Crocin	Doxorubicin-induced myocardial toxicity	Reduces oxidative stress, enhances host anti-oxidant defenses and decreases apoptosis by restoring the balance between proinflammatory (TNF-α, IL-1β and caspase-3) and antiinflammatory (IL-10) cytokines.	Elsherbiny *et al*., 2016
Hesperetin	Doxorubicin-induced testicular toxicity	Prevents oxidative stress, DNA damage and apoptosis by reducing expression of NF-κβ, p38 and caspase-3.	Trivedi *et al*., 2011
Edarabone	Doxorubicin-induced cardiomyopathy	Improves conduction abnormalities, arrhythmia and myocardial ischemia	Xin *et al*., 2011
Diphenylmethyl selenocyanate (DMSE)	Cisplatin-induce nephrotoxicity	Enhances activity of antioxidant enzymes and inhibits expression of proinflammatory COX-2 and *i*NOS.	Chakraborty *et al*., 2011

Contd…

Table 3: Promising preclinical chemoprotective compounds (Contd…)

Compounds	Chemoprotective efficacy	Mechanism of action	References
Erdosteine	Cisplatin-induced renal failure	Modulates function of hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH)	Yilmaz *et al*., 2004
Vanadium(III)-L-cysteine	Cisplatin-induced nephrotoxicity, myelosuppression and genotoxicity	Restores host redox status, induces Nrf2-mediated ARE pathway and inhibits expression of NFκβ and IL-6.	Basu *et al*., 2015; 2016
Lycopene	Cisplatin-induced nephrotoxicity	Stimulates Nrf2/HO-1 signaling pathway and inhibits NFκβ expression	Sahn *et al*., 2010
Ginseng	Cisplatin-induced nephropathy	Enhances expression of p53 and cJNK followed by reduction in the expression of caspase-3	Park *et al*., 2015
Eicosapentaenoic acid and Docosahexaenoic acid	Cisplatin-induced testicular and spermatological damage	Attenuates oxidative stress by restoring antioxidant defense system	Ciftci *et al*., 2014
Curcumin	Protects normal organs including liver, kidney, oral mucosa, and heart from chemotherapy-induced toxicity	Induces activation of Nrf2 and upregulates expression of antioxidant enzymes. Quenches free radicals and inhibits p300 HAT activity.	Goel and Aggarwal, 2010
Facteur thymique serique (FTS)	Bleomycin-induced pulmonary fibrosis	Suppresses local synthesis of proinflammatory cytokines – TNF-α and IL-1β, chemokines – MCP-1, MIP-1α RANTES, MIP-2 and KC	Yara *et al*., 2001

chemoprotectants there are also some compounds which show promising chemoprotective efficacy in preclinical stages (Table 3).

Conclusion

Evidences in literature validate the potential role of chemoprotectants in the management of toxicities encountered by patients receiving cytotoxic chemotherapeutic drugs. Several of the compounds provide protection without interference with the antitumor activity of the administered antineoplastic agents, and may enable delivery of higher doses of chemotherapeutics. The chemoprotectants in combination with chemotherapeutics is partially effective due to moderate protective efficacy towards normal tissues, potential risk of tumor growth and adverse reactions. The therapy in cancer may have to be directed to develop novel chemoprotective compounds with enhanced specificity to normal cells, with delivery of the drugs not affecting the antitumor efficacy of cytotoxic agents. Development of such selective chemoprotective agents that lessen the burden of treatment and are cost effective is the need of today.

Conflict of Interest

No conflict of interest declaration.

Acknowledgement

The authors acknowledge Prof. (Dr.) Jaydip Biswas, Director, Chittaranjan National Cancer Institute, for his support during the study. The authors also acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi for Research Associateship (09/030(0075)/2015 EMR-I) to Abhishek Basu; Indian Council of Medical Research (ICMR), New Delhi for Senior

REFERENCES

Aapro M, Carides A, Rapoport BL, Schmoll HJ, Zhang L, Warr D. Aprepitant and fosaprepitant: a 10-year review of efficacy and safety. *Oncologist* 2015;20(4):450–458.

Aapro M, Rugo H, Rossi G, Rizzi G, Borroni ME, Bondarenko I, *et al*. A randomized phase III study evaluating the efficacy and safety of NEPA, a fixed-dose combination of netupitant and palonosetron, for prevention of chemotherapy-induced nausea and vomiting following moderately emetogenic chemotherapy. *Ann Oncol* 2014;25(7):1328–1333.

Aapro MS, Schmoll HJ, Jahn F, Carides AD, Webb RT. Review of the efficacy of aprepitant for the prevention of chemotherapy-induced nausea and vomiting in a range of tumor types. *Cancer Treat Rev*

2013;39(1):113–117.

Adams VR, Brenner TL. Oprelvekin (Neumega®). *J Oncol Pharm Practice* 1999;5(3):117–124.

Ali BH, Al Moundhri MS. Agents ameliorating or augmenting the nephrotoxicity of cisplatin and other platinum compounds: a review of some recent research. *Food Chem Toxicol* 2006;44(8):1173–1183.

Altayli E, Malkoc E, Alp BF, Korkmaz A. Prevention and treatment of cyclophosphamide and ifosfamide-induced hemorrhagic cystitis. *J Mol Pathophysiol* 2012;1(1):53–62.

Arellano ML, Langston A, Winton E, Flowers CR, Waller EK. Treatment of relapsed acute leukemia after allogeneic transplantation: a single center experience. *Biol Blood Marrow Transplant* 2007;13(1):116–123.

Bailey MM, Sawyer RD, Behling JE, Boohaker JG, Hicks JG, O'donnell MA, Stringer KR, Rasco JF, Hood RD. Prior exposure to indole-3-carbinol decreases the incidence of specific cyclophosphamide-induced developmental defects in mice. *Birth Defects Res B Dev Reprod Toxicol* 2005;74(3):261–267.

Baldo BA. Side effects of cytokines approved for therapy. *Drug Saf* 2014;37(11):921–943.

Basch E, Hesketh PJ, Kris MG, Prestrud AA, Temin S, Lyman GH. Antiemetics: american society of clinical oncology clinical practice guideline update. *J Oncol Pract* 2011;7(6):395–398.

Basu A, Ghosh P, Bhattacharjee A, Patra AR, Bhattacharya S. Prevention of myelosuppression and genotoxicity induced by cisplatin in murine bone marrow cells: effect of an organovanadium compound vanadium(III)-L-cysteine. *Mutagenesis* 2015;30(4):509–517.

Basu A, Singha Roy S, Bhattacharjee A, Bhuniya A, Baral R, Biswas J, Bhattacharya S. Vanadium(III)-L-cysteine protects cisplatin-induced nephropathy through activation of Nrf2/HO-1 pathway. *Free Radic Res* 2016;50(1):39–55.

Bergström M, Hargreaves RJ, Burns HD, Goldberg MR, Sciberras D, Reines SA, *et al*. Human positron emission tomography studies of brain neurokinin 1 receptor occupancy by aprepitant. *Biol Psychiatry* 2004;55(10):1007–1012.

Berl T, Schwertschlag U. Preclinical pharmacologic basis for clinical use of rhIL-11 as an effective platelet-support agent. *Oncology (Williston Park)* 2000;14(9 Suppl 8):12–20.

Beveridge RA, Miller JA, Kales AN, Binder RA, Robert NJ, Harvey JH, *et al*. A comparison of efficacy of sargramostim (yeast-derived RhuGM-CSF) and filgrastim (bacteria-derived RhuG-CSF) in the therapeutic setting of chemotherapy-induced myelosuppression. *Cancer Invest* 1998;16(6):366–373.

Bhattacharjee A, Basu A, Biswas J, Bhattacharya S. Nano-Se attenuates cyclophosphamide-induced pulmonary injury through modulation of oxidative stress and DNA damage in Swiss albino mice. *Mol Cell Biochem* 2015;405(1-2):243–256.

Bhattacharjee A, Basu A, Ghosh P, Biswas J, Bhattacharya S. Protective effect of Selenium nanoparticle against cyclophosphamide induced hepatotoxicity and genotoxicity in Swiss albino mice. *J*

Biomater Appl 2014;29(2):303–317.

Bhattacharya A. Methylselenocysteine: a promising antiangiogenic agent for overcoming drug delivery barriers in solid malignancies for therapeutic synergy with anticancer drugs. *Expert Opin Drug Deliv* 2011;8(6):749–763.

Boyer MW, Waller EK, Bray RA, Unangst T, Johnson TS, Phillips C, *et al*. Cytokine upregulation of the antigen presenting function of acute myeloid leukemia cells. Leukemia. 2000;14(3):412–418.

Braun MS, Seymour MT. Balancing the efficacy and toxicity of chemotherapy in colorectal cancer. *Ther Adv Med Oncol* 2011;3(1):43–52.

Cantor SB, Elting LS, Hudson DV Jr, Rubenstein EB. Pharmacoeconomic analysis of oprelvekin (recombinant human interleukin-11) for secondary prophylaxis of thrombocytopenia in solid tumor patients receiving chemotherapy. *Cancer* 2003;97(12):3099–3106.

Carr AC, Vissers MC, Cook JS. The effect of intravenous vitamin C on cancer- and chemotherapy-related fatigue and quality of life. *Front Oncol* 2014;4:283.

Chakraborty P, Roy SS, Sk UH, Bhattacharya S. Amelioration of cisplatin-induced nephrotoxicity in mice by oral administration of diphenylmethyl selenocyanate. *Free Radic Res* 2011;45(2):177–187.

Chaveli-López B, Bagán-Sebastián JV. Treatment of oral mucositis due to chemotherapy. *J Clin Exp Dent* 2016;8(2):e201–e209.

Ciftci O, Cetin A, Aydin M, Kaya K, Oguz F. Fish oil, contained in eicosapentaenoic acid and docosahexaenoic acid, attenuates testicular and spermatological damage induced by cisplatin in rats. *Andrologia* 2014;46(10):1161–1168.

Conklin KA. Cancer chemotherapy and antioxidants. *J Nutr* 2004;134(11):3201S–3204S.

Devine A, Marignol L. Potential of Amifostine for Chemoradiotherapy and Radiotherapy-associated Toxicity Reduction in Advanced NSCLC: A Meta-Analysis. *Anticancer Res* 2016;36(1):5–12.

DeVita VT, Chu E. A History of Cancer Chemotherapy. *Cancer Res* 2008;68(21):8643–8653.

Di Maio M, Bria E, Banna GL, Puglisi F, Garassino MC, Lorusso D, *et al*. Prevention of chemotherapy-induced nausea and vomiting and the role of neurokinin 1 inhibitors: from guidelines to clinical practice in solid tumors. *Anticancer Drugs* 2013;24(2):99–111.

Dorner AJ, Goldman SJ, Keith Jr JC. Interleukin-11: biological activity and clinical studies. *Biodrugs* 1997;8:418–429.

Du X, Williams DA. Interleukin-11: review of molecular, cell biology, and clinical use. *Blood* 1997;89:3897–3908.

Elsherbiny NM, Salama MF, Said E, El-Sherbiny M, Al-Gayyar MM. Crocin protects against doxorubicin-induced myocardial toxicity in rats through down-regulation of inflammatory and apoptic pathways. *Chem Biol Interact* 2016;247:39–48.

Finch PW, Mark Cross LJ, McAuley DF, Farrell CL. Palifermin for the protection and regeneration of epithelial tissues following injury: new findings in basic research and pre-clinical models. *J Cell Mol Med*

2013;17(9):1065–1087.

Gaurav K, Goel RK, Shukla M, Pandey M. Glutamine: A novel approach to chemotherapy-induced toxicity. *Indian J Med Paediatr Oncol* 2012;33(1):13–20.

Gautam A, Gupta A, Lomash V, Pant SC, Vijayaraghavan R. Prophylactic efficacy of combination of DRDE-07 and its analogues with amifostine against sulphur mustard induced systemic toxicity. *Indian J Exp Biol* 2010;48(7):752–761.

Goel A, Aggarwal BB. Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. *Nutr Cancer* 2010;62(7):919–930.

Gómez Raposo C, Pinto Marín A, González Barón M. Colony-stimulating factors: clinical evidence for treatment and prophylaxis of chemotherapy-induced febrile neutropenia. *Clin Transl Oncol* 2006;8(10):729–734.

Grunberg SM, Slusher B, Rugo HS. Emerging treatments in chemotherapy-induced nausea and vomiting. *Clin Adv Hematol Oncol*. 2013;11(2 Suppl 1):1–18.

Haas R, Murea S. The role of granulocyte colony-stimulating factor in mobilization and transplantation of peripheral blood progenitor and stem cells. *Cytokines Mol Ther* 1995;1(4):249–270.

Hargreaves R, Ferreira JC, Hughes D, Brands J, Hale J, Mattson B, *et al*. Development of aprepitant, the first neurokinin-1 receptor antagonist for the prevention of chemotherapy-induced nausea and vomiting. *Ann N Y Acad Sci* 2011;1222:40–48.

Hasinoff BB, Hellmann K, Herman EH, Ferrans VJ. Chemical, biological and clinical aspects of dexrazoxane and other bisdioxopiperazines. *Curr Med Chem* 1998;5(1):1–28.

Henk HJ, Li X, Becker LK, Xu H, Gong Q, Deeter RG, *et al*. Comparative effectiveness of colony-stimulating factors in febrile neutropenia prophylaxis: how results are affected by research design. *J Comp Eff Res* 2015;4(1):37–50.

Hershman DL, Lacchetti C, Dworkin RH, Lavoie Smith EM, Bleeker J, Cavaletti G, *et al*, American Society of Clinical Oncology. Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers: American Society of Clinical Oncology clinical practice guideline. *J Clin Oncol* 2014;32(18):1941–1967.

Hoekman K, van der Vijgh WJ, Vermorken JB. Clinical and preclinical modulation of chemotherapy-induced toxicity in patients with cancer. *Drugs* 1999;57(2):133–155.

Jena G, Vikram A, Tripathi DN, Ramarao P. Use of chemoprotectants in chemotherapy and radiation therapy: the challenges of selecting an appropriate agent. *Integr Cancer Ther* 2010;9(3):253–258.

Jones RL. Utility of dexrazoxane for the reduction of anthracycline-induced cardiotoxicity. *Expert Rev Cardiovasc Ther* 2008;6(10):1311–1317.

Kane RC, McGuinn WD Jr, Dagher R, Justice R, Pazdur R. Dexrazoxane (Totect): FDA review and approval for the treatment of accidental extravasation following intravenous anthracycline chemotherapy.

Oncologist 2008;13(4):445–450.

Kaye JA. FDA licensure of Neumega® to prevent severe chemotherapy-induced thrombocytopenia. *Stem Cells* 1998;16(suppl 2):207–223

Kelsey PJ, Oliveira MC, Badoglio M, Sharrack B, Farge D, Snowden JA. Haematopoietic stem cell transplantation in autoimmune diseases: From basic science to clinical practice. *Curr Res Transl Med* 2016;64(2):71–82.

Kemp G, Rose P, Lurain J, Berman M, Manetta A, Roullet B, *et al*. Amifostine pretreatment for protection against cyclophosphamide-induced and cisplatin-induced toxicities: results of a randomized control trial in patients with advanced ovarian cancer. *J Clin Oncol* 1996;14(7):2101–2112.

Khaw SL, Downie PA, Waters KD, Ashley DM, Heath JA. Adverse hypersensitivity reactions to mesna as adjunctive therapy for cyclophosphamide. *Pediatr Blood Cancer* 2007;49(3):341–343.

Kouvaris JR, Kouloulias VE, Vlahos LJ. Amifostine: the first selective-target and broad-spectrum radioprotector. *Oncologist* 2007;12(6):738–747.

Kreidieh FY, Moukadem HA, El Saghir NS. Overview, prevention and management of chemotherapy extravasation. *World J Clin Oncol* 2016;7(1):87–97.

Langford P, Chrisp P. Fosaprepitant and aprepitant: an update of the evidence for their place in the prevention of chemotherapy-induced nausea and vomiting. *Core Evid* 2010;5:77–90.

Links M, Lewis C. Chemoprotectants: a review of their clinical pharmacology and therapeutic efficacy. *Drugs* 1999;57(3):293–308.

Liu B, Ezeogu L, Zellmer L, Yu B, Xu N, Joshua Liao D. Protecting the normal in order to better kill the cancer. *Cancer Med* 2015;4(9):1394–1403.

Marx GM, Friedlander ML. Drug toxicity prevention and management. *CME J Gynecol Oncol* 2010;18:29–33.

Mehta HM, Malandra M, Corey SJ. G-CSF and GM-CSF in Neutropenia. *J Immunol* 2015;195(4):1341–1349.

Nakayama A, Alladin KP, Igbokwe O, White JD. Systematic review: generating evidence-based guidelines on the concurrent use of dietary antioxidants and chemotherapy or radiotherapy. *Cancer Invest* 2011;29(10):655–667.

Nicolatou-Galitis O, Sarri T, Bowen J, Di Palma M, Kouloulias VE, Niscola P, *et al*; Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO). Systematic review of amifostine for the management of oral mucositis in cancer patients. *Support Care Cancer* 2013;21(1):357–364.

Panjwani M. Efficacy of palifermin in the hematopoietic stem cell transplant setting. *J Adv Pract Oncol* 2013;4(2):89–100.

Park JY, Choi P, Kim T, Ko H, Kim HK, Kang KS, Ham J. Protective Effects of Processed Ginseng and Its Active Ginsenosides on Cisplatin-Induced Nephrotoxicity: In Vitro and in Vivo Studies. *J Agric Food Chem* 2015;63(25):5964–5969.

Payne H, Adamson A, Bahl A, Borwell J, Dodds D, Heath C, *et al*. Chemical- and radiation-

induced haemorrhagic cystitis: current treatments and challenges. *BJU Int* 2013;112(7):885–897.

Popelová O, Sterba M, Simůnek T, Mazurová Y, Guncová I, Hroch M, *et al*. Deferiprone does not protect against chronic anthracycline cardiotoxicity in vivo. *J Pharmacol Exp Ther* 2008;326(1):259–269.

Radomska-Leśniewska DM, Skopiński P. N-acetylcysteine as an anti-oxidant and anti-inflammatory drug and its some clinical applications. *Centr Eur J Immunol* 2012;37(1):57–66.

Sahin K, Tuzcu M, Sahin N, Ali S, Kucuk O. Nrf2/HO-1 signaling pathway may be the prime target for chemoprevention of cisplatin-induced nephrotoxicity by lycopene. *Food Chem Toxicol* 2010;48(10):2670–2674.

Sai Sampath T, Kanaka Durga M, Saranya Bh, Kalyani K. Review on plant derived natural products and their analogues with chemoprotective activity against genotoxicity of cyclophosphamide. *Int J Pharma Bio Sci* 2011;2(3):375–386.

Salman D, Swinden J, Barton S, Peron JM, Nabhani-Gebara S. Evaluation of the stability profile of anticancer drugs: A review of Ifosfamide and Mesna regimen for the treatment of metastatic soft tissue sarcoma. *J Oncol Pharm Pract* 2016;22(1):86–91.

Sathe NA, Krishnaswami S, Andrews J, Ficzere C, McPheeters ML. Pharmacologic Agents That Promote Airway Clearance in Hospitalized Subjects: A Systematic Review. *Respir Care* 2015;60(7):1061–1070.

Savoye C, Swenberg C, Hugot S, Sy D, Sabattier R, Charlier M, *et al*. Thiol WR-1065 and disulphide WR-33278, two metabolites of the drug ethyol (WR-2721), protect DNA against fast neutron-induced strand breakage. *Int J Radiat Biol* 1997;71(2):193–202.

Seely D, Wu P, Fritz H, Kennedy DA, Tsui T, Seely AJ, *et al*. Melatonin as adjuvant cancer care with and without chemotherapy: a systematic review and meta-analysis of randomized trials. *Integr Cancer Ther* 2012;11(4):293–303.

Sehested M, Jensen PB, Sørensen BS, Holm B, Friche E, Demant EJ. Antagonistic effect of the cardioprotector (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187) on DNA breaks and cytotoxicity induced by the topoisomerase II directed drugs daunorubicin and etoposide (VP-16). *Biochem Pharmacol* 1993;46(3):389–393.

Sitaraman SV, Gewirtz AT. Oprelvekin. Genetics Institute. *Curr Opin Investig Drugs* 2001;2(10):1395–1400.

Smith JW. Tolerability and side-effect profile of rhIL-11. *Oncology* 2000;14(9 Suppl 8):41–47.

Sourgens H, Lefrère F. A systematic review of available clinical evidence - filgrastim compared with lenograstim. *Int J Clin Pharmacol Ther* 2011;49(8):510–518.

Tatlidede E, Sehirli O, Velioğlu-Oğünc A, Cetinel S, Yeğen BC, Yarat A, Süleymanoğlu S, Sener G. Resveratrol treatment protects against doxorubicin-induced cardiotoxicity by alleviating oxidative damage. *Free Radic Res* 2009;43(3):195–205.

Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, *et al*. Role of glutathione in cancer progression and chemoresistance. *Oxid Med Cell Longev*

2013;2013:972913.

Vadhan-Raj S, Goldberg JD, Perales MA, Berger DP, van den Brink MR. Clinical applications of palifermin: amelioration of oral mucositis and other potential indications. *J Cell Mol Med* 2013;17(11):1371–1384.

van den Berg JH, Beijnen JH, Balm AJ, Schellens JH. Future opportunities in preventing cisplatin induced ototoxicity. *Cancer Treat Rev* 2006;32(5):390–397.

Waller EK. The role of sargramostim (rhGM-CSF) as immunotherapy. *Oncologist* 2007;12 Suppl 2:22–26.

Wilde MI, Faulds D. Oprelvekin: a review of its pharmacology and therapeutic potential in chemotherapy-induced thrombocytopenia. *BioDrugs* 1998;10(2):159–171.

Xin Y, Zhang S, Gu L, Liu S, Gao H, You Z, Zhou G, Wen L, Yu J, Xuan Y. Electrocardiographic and biochemical evidence for the cardioprotective effect of antioxidants in acute doxorubicin-induced cardiotoxicity in the beagle dogs. *Biol Pharm Bull* 2011;34(10):1523–1526.

Yara S, Kawakami K, Kudeken N, Tohyama M, Teruya K, Chinen T, Awaya A, Saito A. FTS reduces bleomycin-induced cytokine and chemokine production and inhibits pulmonary fibrosis in mice. Clin Exp Immunol. 2001;124(1):77–85.

Yilmaz HR, Iraz M, Sogut S, Ozyurt H, Yildirim Z, Akyol O, Gergerlioglu S. The effects of erdosteine on the activities of some metabolic enzymes during cisplatin-induced nephrotoxicity in rats. *Pharmacol Res* 2004;50(3):287–290.

Zhang J, Tian Q, Zhou S. Clinical Pharmacology of Cyclophosphamide and Ifosfamide. *Current Drug Therapy* 2006;1:55–84.

Zhang S, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 2012;18(11):1639–1642.

Review

BRJ
Biomedical Research Journal

Oral Feeding With Arachidonic Acid (AA) and Docosahexanoic Acid (DHA) Help in Better Recovery of Haematopoiesis in Sub-lethally Irradiated Mice

Kedar Limbkar, Vaijayanti Kale and Lalita Limaye*

Stem Cell Laboratory, National Centre for Cell Science, University of Pune Campus, Pune – 411007, India

Haematopoiesis is severely hampered after exposure to ionizing radiations. Role of polyunsaturated fatty acids (PUFAs) during embryonic development as well as during various physiological processes is well established. However, few studies on their effect on haematopoiesis are reported. Hence, we studied the effect of oral administration of PUFAs-AA/DHA on haematopoiesis of sub-lethally irradiated mice. To determine the optimal dose for haematopoiesis, non-irradiated healthy mice were orally fed with different doses of AA/DHA daily for ten days. Additionally, mice were sub lethally irradiated and kept for ten days on normal diet. Further, sub-lethally irradiated mice were orally fed with optimal dose of AA/DHA for ten days. Mice from the experiments were sacrificed after ten days and their bone marrow cells were harvested and analyzed for their total nucleated cell (TNC) count, side population (SP) and $lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$(LSK) phenotype. Peripheral blood collected from this set of mice was subjected to hemogram analysis. Daily dose of 8 mg AA/DHA for ten days was assessed as optimal for enhancing BM-MNCs and primitive HSCs in non-irradiated mice. Significant depletion in BM-MNCs, SP and LSK cells was observed in sub lethally irradiated mice compared to un-irradiated control mice. Feeding with DHA or AA in sub lethally irradiated mice showed significantly higher number of BM-MNCs and increased percentage of SP and LSK cells, suggesting that DHA and AA resulted in better recovery of hematopoietically compromised mice. The data indicated that DHA or AA may serve as useful dietary supplements in patients exposed to irradiation.

INTRODUCTION

Exposure to ionizing radiations is common in the modern age as they are widely used in research, diagnosis, manufacturing and construction (Brenner *et al.,* 2007). Ionizing radiation is a common modality of treatment of cancer patients. Haematopoiesis maintains blood cell lineages at constant level. Bone marrow provides a favorable microenvironment for hematopoietic stem cells, enabling repopulation, differentiation and migration, and also regulates generation of blood cells (Shen *et al.,* 2010). The extremely proliferative

Key words: HSCs, haematopoiesis, radiation injury, docosahexanoic acid, arachidonic acid, recovery.
***Corresponding Author:** Lalita Limaye, Stem Cell Laboratory, National Centre for Cell Science, NCCS complex, University of Pune Campus, Ganeshkhind, Pune – 411007, Maharashtra, India.
Email: lslimaye@nccs.res.in

property of the HSCs (Ogawa M., 1993) required to maintain homeostasis makes them highly radiosensitive (Chinsoo *et al.,* 1998; Chitteti *et al.,* 2011; Till *et al.,* 1964). A radiation dose of 2-8 Gy may create significant damage to the bone marrow causing the hematopoietic syndrome of the acute radiation syndrome (HS-ARS), characterized by life-threatening lymphocytopenia, neutropenia, and thrombocytopenia, and possible death due to infection and/or bleeding (Anno *et al.,* 1989; Coleman *et al.,* 2001; Simopoulos AP, 2002).

Diet plays a key role in normal functioning and development. ω-3 (n-3) and ω-6 (n-6) polyunsaturated fatty acids (PUFAs) are important structural and functional components of cell membrane phospholipids. These form the essential fatty acids, as they cannot be synthesized in the human body and must be obtained from diet (Gebauer *et al.,* 2006). As essential nutrients obtained only through dietary intake, their tissue content in individuals can vary, but may be modified through dietary intervention. The beneficial effects of DHA and AA are observed in humans and animal models of diabetes, obesity, cancer, hypertension, autoimmune disorders, mental health, and cardiovascular diseases, etc. They play an important role in embryonic development, development of vision and neuronal development. The metabolites play key role in cell signaling, and thereby modulate various physiological and pathological processes (Belluzzi *et al.,* 1996; Ismail HM, 2005; Shannon *et al.,* 2007; Simopoulos, 2009). PUFAs also get incorporated in membrane lipid raft, consequently altering the membrane composition (Turk *et al.,* 2013). These lipid rafts have important role in embryonic stem cell self-renewal (Lee *et al.,* 2010). The metabolism of AA/DHA is depicted in the flow chart below (Fig. 1). AA is broken down to either leukotrienes, prostaglandins or eicosatetraenoic acids by lipoxygenases, cyclooxygenases and cytochrome P450, respectively. Similarly, DHA is metabolized through lipoxygenases to resolvins.

The effect of PUFAs on haematopoiesis is complex, since these fatty acids are processed into leukotrienes, eicosanoids and prostaglandins, which independently affect haematopoiesis. Several reports suggest that the PUFAs act on human marrow myelopoiesis and erythropoiesis as evidenced by the growth of committed progenitors (CFU-GM and BFU-E) in

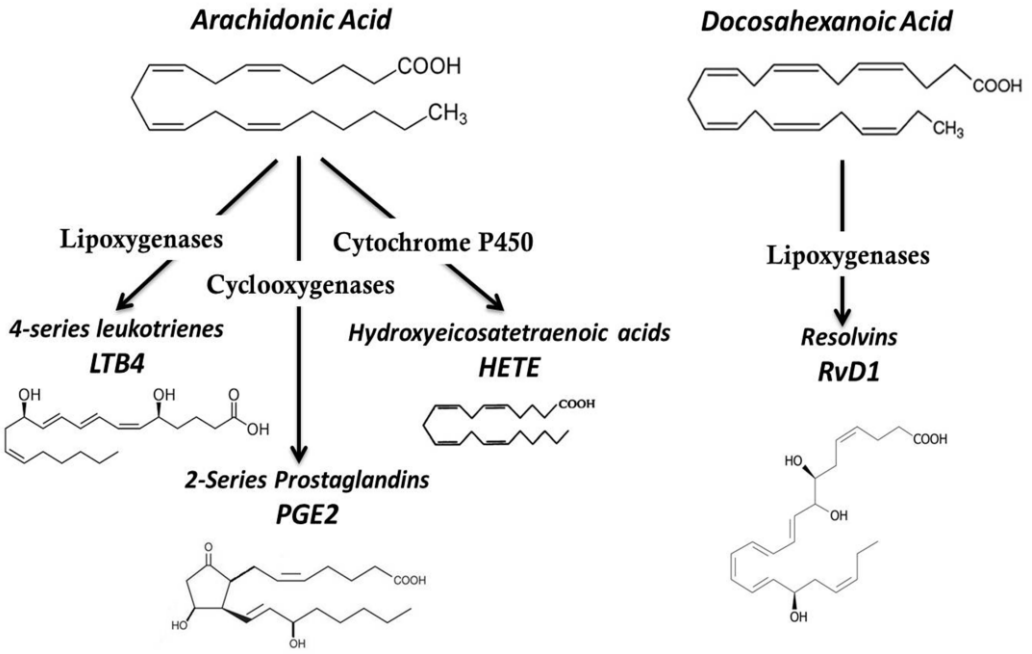


Figure 1: Flow chart showing metabolism of AA/DHA.

vitro (Dupuis *et al.,* 1997). It has been reported that diet rich in n-3 PUFAs relative to the proportion of n-6 PUFAs, affects myelopoiesis by reducing total myeloid progenitor cell frequency and promotes differentiation of specific progenitor cell types in the bone marrow of mice (Verny *et al.,* 2009). Besides, AA and DHA influences megakaryopoiesis and thrombopoiesis *in vitro* (Shabrani *et al.,* 2012; Siddqui *et al.,* 2011).

Thus, our hypothesis was 'whether oral feeding of PUFAs in hematopoietically compromised mice, enhances haematopoiesis in mice'. In the present study, we demonstrate that feeding sub-lethally irradiated mice with DHA or AA orally for ten days enhances the bone marrow cell count and increases haematopoiesis.

MATERIALS AND METHODS

Mice

Protocols used in the animal experimentation were approved by the Institutional Animal Ethics Committee (IAEC). C57BL/6 mice (6–8 weeks old, females) were used for the feeding experiments.

Nutraceuticals

Docosahexanoic acid (> 99% Pure) and Arachidonic acid (> 99% Pure) were procured from NuChek Prep (Elysian, USA).

Oral Feeding of Mice With Nutraceuticals

The following protocol was followed: 1) Mice were fed various doses of AA/DHA: 2, 4, 8 and 16 mg. Control mice were fed with PBS (vehicle control). 2) Control and test mice were subjected to dose of 4.5 Gy irradiation using ^{60}Co Gamma Chamber (BRIT, Mumbai, India) and kept on normal diet for ten days. Non-irradiated mice were kept as control. 3) Control mice and test mice were sub-lethally irradiated as described above. Test mice in addition to normal solid feed were fed 8 mg AA/DHA daily through oral feeding gavage in separate sets for ten days. Mice fed with PBS (henceforth will be referred as unfed) were used as control.

Harvesting and Processing of BM and PBL

Mice from all experiments were sacrificed after ten days and their bone marrow mononuclear cells (BM-MNCs) were harvested by flushing tibia and femur bones with 21G syringe. Total nucleated cells (TNCs) were counted manually using hemocytometer after mixing them with Turk's solution containing crystal violet and acetic acid. They were further subjected to flow cytometry analysis of HSCs like SP and LSK analysis.

Blood was collected from mice that were irradiated and then fed with PBS/AA/DHA and was subjected to hemogram analysis using automated blood cell counter.

Side Population (SP) Analysis

SP analysis was performed as described by Eaker *et al*. (Eaker *et al*., 2004). Briefly, 10^6 BM MNCs of fed or unfed mice were stained with 5 µg Hoechst 33342(Sigma), with or without 50 µM Verapamil (Sigma Aldrich, St Louis, USA), for 90 min at 37°C. The cells were stained with 50 µM Propidium Iodide (PI) for detecting dead cells. The cells were analyzed on a flow cytometer (FACS ARIA III SORP, Becton Dickinson) using UV laser.

Phenotypic Analysis

LSK analysis was performed as per Uchida *et al.* (Uchida *et al.*, 1992). Briefly, 10^6 BM MNCs were suspended in IMDM containing 20% FBS. The cells were washed and suspended in PBS containing 0.1% BSA and 0.1% sodium azide, and stained with c-Kit CD117-PE-Cy7, CD45.2-PB, lineage marker cocktail (CD3e, CD11b, CD45R/B220,

Ly-76, Ly-6G, and Ly-6C)-APC, Sca-1/Ly-6A/E-PE (BD Bioscience, San Diego, USA), at 4°C for 45 min with frequent mixing. The cells were washed with PBS and fixed in 1% buffered paraformaldehyde. Appropriate isotype controls were used. Fifty thousand events in the lineage negative gate were acquired for each sample (FACS Canto II; BD Bioscience, San Diego, USA). The flow cytometry data was analyzed using FACS Diva™ (BD Bioscience) software. c-Kit and Sca-1 double positive population was gated in lineage negative cells to get LSK population.

Statistical Analysis

Statistical analysis was done using Sigma Plot 11 (Jandel Scientific Corporation, San Rafael, California, USA) software using One Way RM-ANOVA. The mean and standard deviation obtained was plotted for the various assays. The data was considered significant if $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). Graphs were plotted using the same software.

RESULTS

Optimal Dose of AA/DHA for Haematopoiesis in Mice

To determine optimal dose of AA or DHA for haematopoiesis, mice were fed for ten days with 2, 4, 8 or 16 mg of AA or DHA, respectively. PBS fed (Unfed) mice were kept as sham control. Mice were sacrificed after 10 days of feeding and their bone marrow mononuclear cells (BM-MNCs) were subjected to total nucleated cell (TNC) count, SP and LSK analysis. As shown in the Fig. 2A, BM MNCs of mice fed with 8 mg AA /DHA showed significantly higher number of TNCs as compared to control mice (PBS fed), indicating 8 mg as the optimal dose. Side population cells are known to give prolonged multi lineage haematopoiesis since they harbor long-term repopulating stem cells. Fig. 2B, shows representative FACS profile of fed and unfed mice for SP cells. Specificity of SP phenotype was confirmed by addition of Verapamil known to abolish SP profile. Cumulative data from five mice indicate that oral dose of 8 mg of AA/ DHA was optimal for stimulating side population (Fig. 2C). LSK cells are known to be primitive stem cells. As observed in Fig. 2E, marrow cells of the AA-fed and DHA fed mice showed higher percentage of LSK cells as compared to the controls. Representative FACS profile is depicted in Fig. 2D. Thus the data show that oral dose of 8 mg of AA or DHA enhances haematopoiesis in mice.

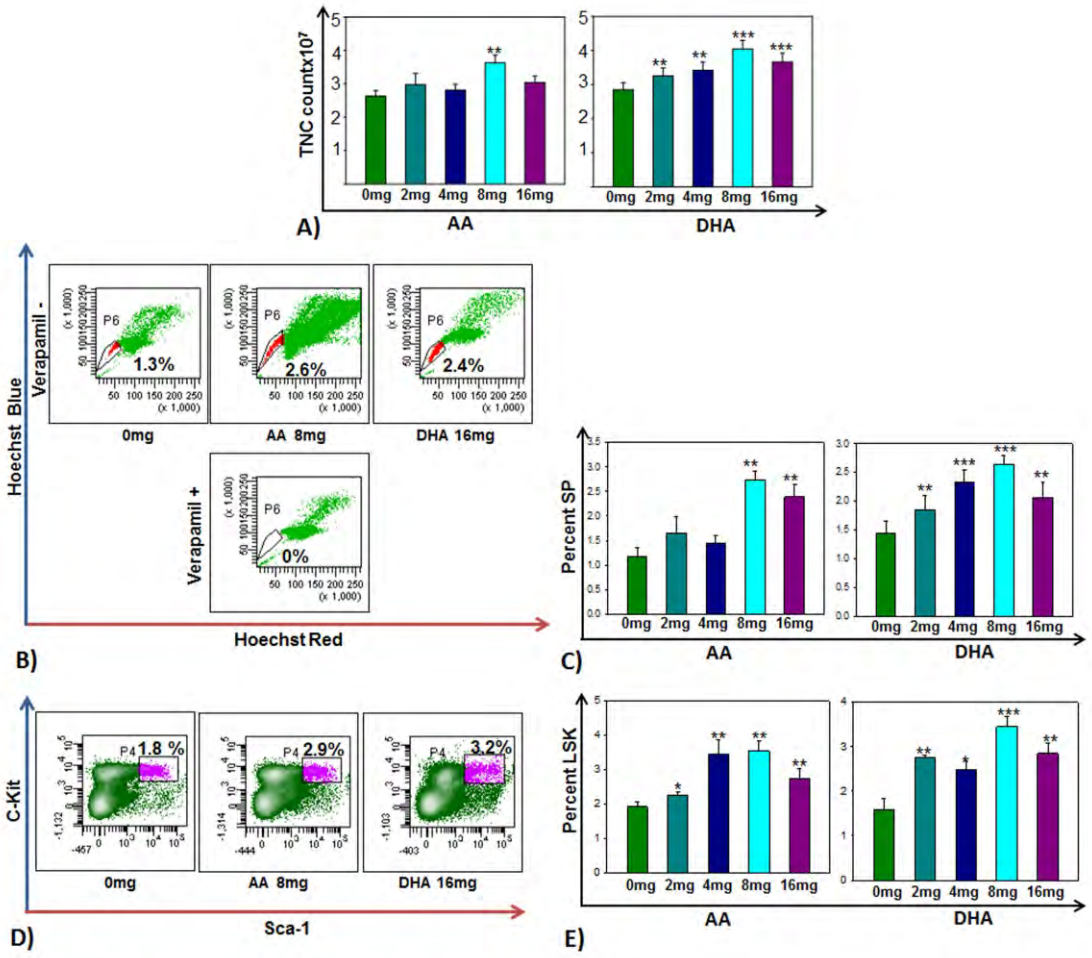


Figure 2. Dose optimization for AA/DHA. To determine optimal dose for haematopoiesis, mice were fed with different doses of AA and DHA daily for ten days. Their BM MNCs were harvested and subjected to various assays for haematopoiesis. Cumulative data from 4 samples clearly shows that dose of 8mg AA/DHA significantly enhanced (A) TNC count; (C) SP; (E) LSK population compared to control. Representative flow cytometry profile from Fig., (B) and (D), depicts the same. N = 5; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Sub-lethal Irradiation Depletes Bone Marrow Cells and Hscs in Mice

To study the effect of irradiation on haematopoiesis, mice were given sub lethal dose (4.5 Gy) of irradiation; healthy, non-irradiated mice were kept as control. Mice were kept untreated for 10 days and sacrificed after 10 days .The bone marrow cells were harvested and analyzed for TNC count, SP cells and LSK cells. Fig. 3A shows that sub lethal irradiation significantly depleted total nucleated cells in mice. Flow cytometry profile (Fig. 3B) and cumulative statistical data in Fig. 3C shows more than two fold reduction in side population cells of irradiated mice. Sub lethal dose of gamma irradiation causes

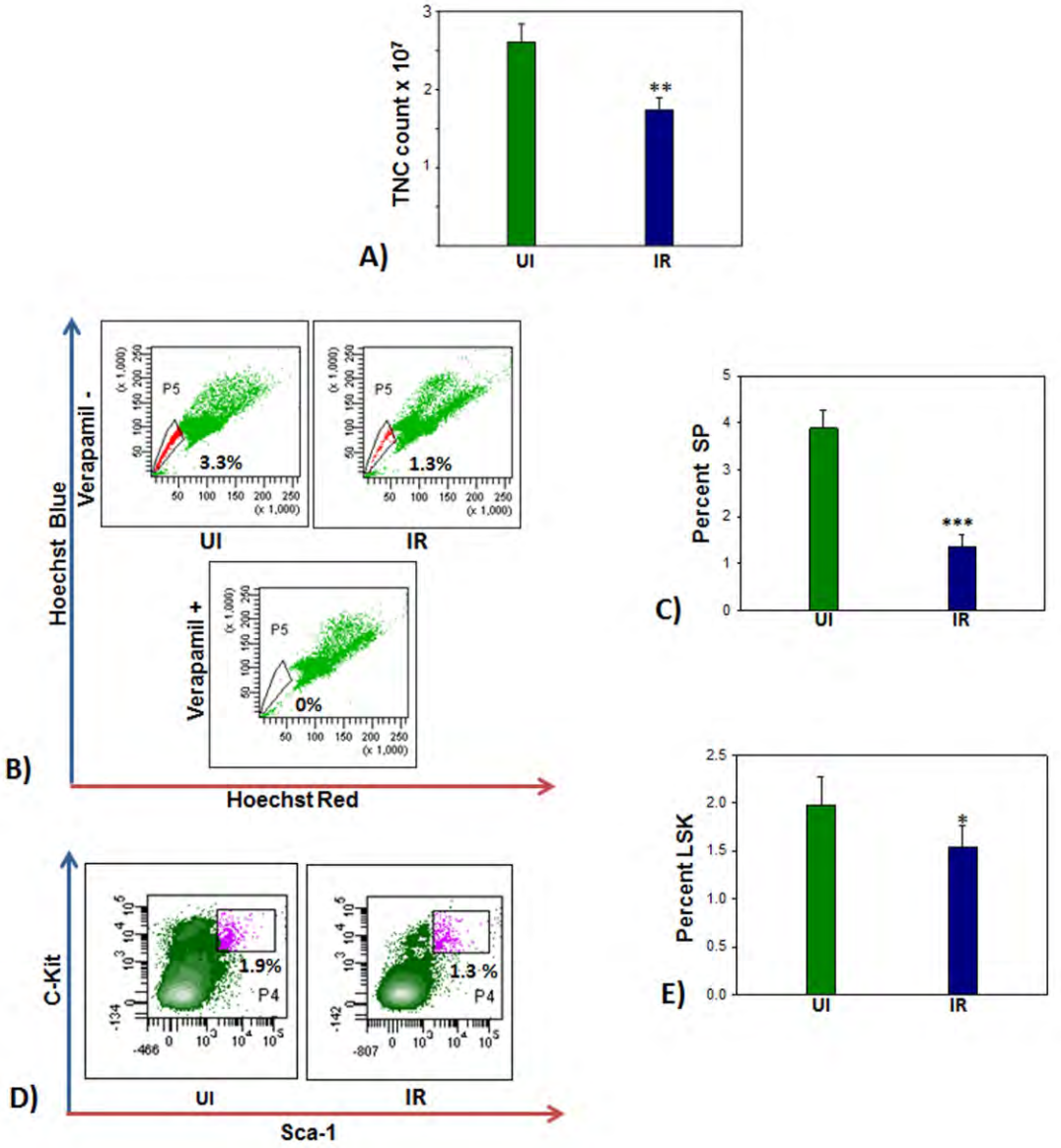


Figure 3. Irradiation hampers haematopoiesis in mice. Mice were sub lethally irradiated and were kept for ten days without any treatment. Non-irradiated mice were kept as control. Mice were sacrificed and their BM MNCs were tested for hematopoiesis. Data clearly shows sub lethal dose of irradiation caused significant decrease in the (A) TNC count, (C) SP and (E) LSK cells in mice. Representative flow cytometry profile of (B) SP and (D) LSK depicts the same. N = 4; *p < 0.05, **p < 0.01, ***p < 0.001.

hematopoietic ablation in mice. Irradiation decreased primitive stem cells as observed by decreased percent LSK (Fig. 3E). Representative flow cytometry profile is depicted in Fig. 3D.

Feeding AA or DHA to Sub-lethally Irradiated Mice Restores Haematopoiesis

Sub-lethally irradiated mice were fed with AA or DHA for ten days. Mice were sacrificed and bone marrow (BM) cells

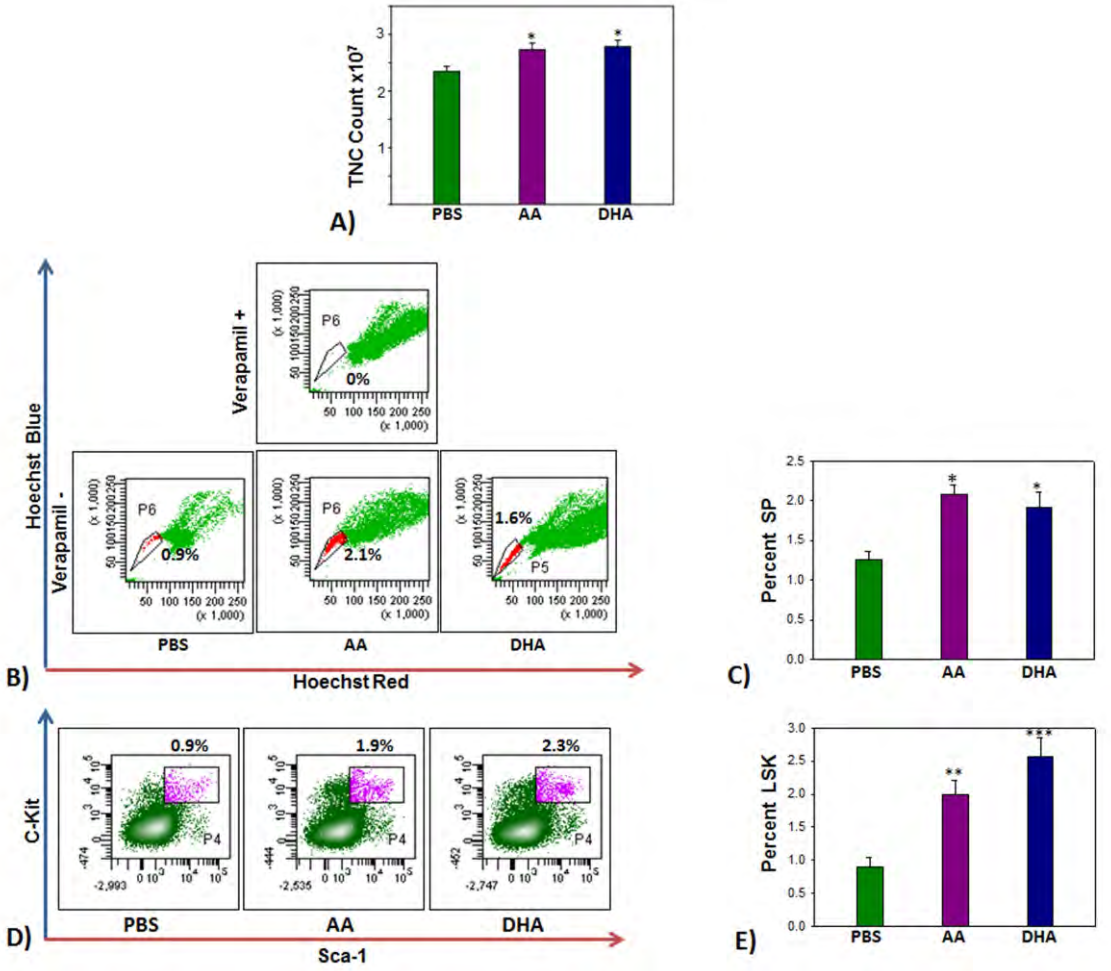


Figure 4. Feeding of AA/DHA restores haematopoiesis in irradiated mice. Mice were given sub lethal dose (4.5 Gy) of irradiation and were fed daily for 10 days with either PBS (control) or 8mg AA /DHA. Mice were sacrificed after 10 days and their BM MNCs were analyzed for hematopoiesis. Irradiated mice, when fed with AA or DHA, showed significant increase in their (A) TNC count, (C) SP and (E) LSK percentage. Flow cytometry profile of one representative sample (B) and (D) also suggests the same. N = 5; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

and peripheral blood (PBL) cells were harvested. Total nucleated cells were increased in BM MNCs of AA or DHA fed mice, as compared to control mice (Fig. 4A). AA/DHA stimulated long term repopulating cells. As shown in Fig. 4B, increased percentage of SP cells was observed in AA (2.1%) and DHA fed mice (1.6%). Cumulative data clearly indicates that AA and DHA caused significant enhancement in the percentage SP in the bone marrow (Fig. 4C). AA and DHA stimulated primitive stem cells. Fig. 4D shows increased percentage of LSK cells in AA fed (1.9%) and DHA fed (2.3%) mice. Significantly increased number of LSK cells were observed in bone marrow of fed mice (Fig. 4E).

Peripheral blood cells of unfed and

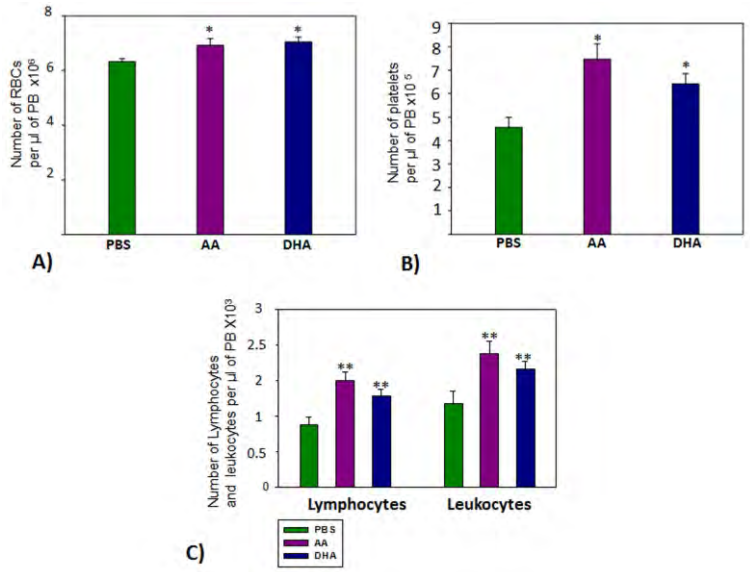


Fig. 5. AA/DHA increased RBC, Platelet and Leukocyte production. Peripheral blood of sub lethally irradiated and fed/unfed mice was subjected to automated blood count analysis. Significant enhancement in (A) RBC number and (B) platelet count was observed in AA/DHA fed mice. AA/DHA feeding also caused significant increase in (C) leukocytes, especially lymphocytes compared to control. N = 5; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

fed mice of this set of experiment were subjected to hemogram analysis. It was observed that feeding of AA and DHA resulted in increased RBC (Fig. 5A) and platelet count (Fig. 5B) in mice. AA and DHA significantly increased leucocytes especially lymphocytes in the peripheral blood of mice (Fig. 5C).

Thus, the data suggests that oral administration of AA or DHA to normal healthy mice stimulates the haematopoiesis. When sub lethally irradiated mice were fed with DHA/AA, it stimulated their long-term repopulating cells, primitive HSCs and promoted enhancement of erythropoiesis and thrombopoiesis.

DISCUSSION

In the present study, we have made a systematic attempt to examine the effect of PUFAs - AA (n6 PUFA) and DHA (n3PUFA) on haematopoiesis of sub lethally irradiated mice. We optimized the dose of the two PUFAs for maximal stimulation of haematopoiesis. Daily oral dose of 8 mg of AA/DHA was beneficial. Our results are accordance with earlier studies. Hoggatt *et al*. (2009) who reported that short-term ex vivo exposure of HSCs to PGE2 -a prostaglandin, derived from AA, enhances their homing, survival and proliferation, resulting in increased long-term repopulating cell (LTRC) and competitive repopulating unit (CRU) frequency. However, the

authors used PGE2 and studied *in vitro* effect on HSCs, whereas we report effect of *in vivo* feeding of purified PUFAs on haematopoiesis. Several studies suggest role of n3PUFAs or n6 PUFAs or their metabolites in stem cell proliferation (Beltz *et al.,* 2007; He *et al.,* 2009; Kawakita *et al.,*2006; Kim *et al.,* 2009; Thangavelu *et al.,* 2007). Our systematic study indicates a direct correlation between oral feeding of AA/DHA and stimulation of haematopoiesis in mice.

Further, we examined the effect of sub-lethal dose of irradiation on haematopoiesis in mice. A reduction in TNC count followed by drastic reduction in SP and LSK cells are hallmark effects of irradiation. Depletion in TNC count may be attributed to hampered self-renewal of HSCs, confirmed by significant reduction in SP cells and LSK percentage. Our data is consistent with earlier reports suggesting that ionizing radiation hampers HSC self-renewal and acute radiation causes BM failure (Hu *et al.,* 2010; Lorrimore *et al.,* 2003; Weiss *et al.,* 2000).

We examined the effect of optimized daily dose of 8 mg of AA/DHA for ten days on sub-lethally irradiated mice and checked their effect on haematopoiesis. We observed significant increase in TNC count, SP cells and LSK cells. Enhancement in haematopoiesis may be because of protective role of PUFAs from radiation injury. Our data are in line with study done by Hoggatt *et al*. (2013), reporting that subcutaneous administration of PGE2 analog, to mice after irradiation, increased their survival by enhancing white blood cells (WBC), polymorphonuclear leukocytes (PMN) and platelets (PLT) over a 30 day period indicating enhanced haematopoietic recovery in mice after irradiation. Gómez de Segura *et al*. (2004) have reported that supplementing the diet with DHA prevented the negative action of 5-FU on mucosal morphometry in rats. Umegaki *et al*. (1997) noted that by feeding mice a diet containing oleic acid before X-ray exposure, experienced greater degrees of immunosuppression (53% and 69%, respectively) than did those consuming diets containing eicosapentaenoic acid alone or in combination with docosahexaenoic acid (DHA) (4% and 24%, respectively). We also observed enhancement in erythropoiesis, thrombopoiesis and leukocytes in PBL of irradiated mice fed with AA/DHA. No significant change in the number of eosinophils, neutrophils, monocytes and granulocytes was observed in PBL of fed

mice (data not shown) suggesting that oral administration of AA/DHA in mice is not causing any lineage bias. Recent study of Xia *et al*. (2015) showed that fish oil-rich diet promotes hematopoiesis in the bone marrow and spleen of mice by increasing TNC count, WBC count and LSK cells in part via the activity of MMP12. However in the study fish oil was mixed with the solid diet of mice. Whereas, we orally administered the defined amount of pure AA/DHA to mice.

Thus, our results demonstrate that oral feeding of AA or DHA enhances haematopoiesis in irradiated mice, and helps in partial recovery from hematopoietic injury. Further studies such as investigating radio-protectant effect of AA/DHA in the context of their ability to quench ROX species and studying mechanism of action of PUFAs will add to their therapeutic application. Dietary interventions of AA or DHA may also enhance stem cell recovery from radiation injury, and hence indicated as an adjunct supplement to radiotherapy, for better recovery of haematopoiesis.

ACKNOWLEDGEMENT

The authors thank Dr. Mande, Director, NCCS, for support; experimental animal facility (EAF) of NCCS for care and maintenance of mice; FACS facility for acquisition of samples, Dr. R. L. Marathe for analysis of PBL samples, Mrs. Nikhat Khan for technical help. We thank Department of Biotechnology (DBT), Government of India, New Delhi for project grant (LL/BT/PR-4930/2013-15), and Indian Council of Medical Research (ICMR), Government of India, New Delhi, for doctoral fellowship of KRL.

CONFLICT OF INTEREST

No conflict of interest.

REFERENCES

Anno GH, Baum SJ, Withers HR, Young RW. Symptomatology of acute radiation effects in humansafter exposure to doses of 0.5–30 Gy. *Health Phys.* 1989;56:821–838.

Belluzzi A, Brignola C, Campieri M, Pera A, Boschi S, Miglioli M. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *N Engl J Med.* 1996; 334:1557–1560.

Beltz BS, Tlusty MF, Benton JL, Sandeman DC. Omega-3 fatty acids upregulate adult neurogenesis. *Neurosci Lett* 2007;415:154–158.

Brenner DJ, Hall EJ. Computed tomography--an increasing source of radiation exposure. *N Engl J Med.* 2007;357(22):2277–2284.

Chinsoo CL, Glatstein E. Radiation Injury. *In*: Fauci AS, Braunwald E, Isselbacher KL,

editors. *Harrison's Principles of Internal Medicine*. McGraw-Hill; New York: 1998. 2548–2565.

Chitteti BR, Kacena MA, Srour EF. Phenotypic characterization of hematopoietic stem cells. In: Broxmeyer HA, editor. *Cord Blood: Biology, Transplantation, Banking, and Regulation.* AABB Press; Bethesda: 2011. 75–85.

Coleman CN, Blakely WF, Fike JR, MacVittie TJ, Metting NF, Mitchell JB, *et al.,* Molecular and cellular biology of moderate-dose (1-10 Gy) radiation and potential mechanisms of radiation protection: report of a workshop at Bethesda, Maryland, December 17-18, 2001. *Radiat Res.* 2003;159(6):812–834.

Dupuis F, Desplat V, Praloran V, Denizot Y. Effects of lipidic mediators on the growth of human myeloid and erythroid marrow progenitors. *J Lipid Mediat Cell Signal.* 1997;16(3):117–125.

Eaker SS, Hawley TS, Ramezani A, Hawley RG. Detection and enrichment of hematopoietic stem cells by side population phenotype. *Methods Mol Biol.* 2004; 263:161–180.

Gebauer Sk, Psota TL, Harris, WS, Kris-Etherton PM. N-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits, *Am J Clin Nutr*; 2006, 83 (Suppl.): 1526–1535.

Gómez de Segura IA, Valderrábano S, Vázquez I, Vallejo-Cremades MT, Gómez-García L, Sánchez M, de Miguel E. Protective effects of dietary enrichment with docosahexaenoic acid plus protein in 5-fluorouracil-induced intestinal injury in the rat. *Eur J Gastroenterol Hepatol*. 2004;16(5):479–485.

He CW, Qu XY, Cui LB, Wang J, Kang JX. Improved spatial learning performance of fat-1 mice is associated with enhanced neurogenesis and neuritogenesis by docosahexaenoic acid. *Proc Natl Acad Sci USA* 2009; 106:11370–11375.

Hoggatt J, Singh P, Sampath J, Pelus LM. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood* 2009; 113:5444–5455.

Hoggatt J, Singh P, Stilger KN, Plett PA, Sampson CH, Chua HL, *et al*. Recovery from hematopoietic injury by modulating prostaglandin E (2) signaling post-irradiation. *Blood Cells Mol Dis* 2013; 50:147–153.

Hu KX, Sun QY, Guo M, Ai HS. The radiation protection and therapy effects of mesenchymal stem cells in mice with acute radiation injury. *Br J Radiol.* 2010;83(985):52–58.

Ismail HM. The role of omega-3 fatty acids in cardiac protection: an overview. *Front Biosci.* 2005; 10:1079–1088.

Kawakita E, Hashimoto M, Shido O. Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. *Neuroscience* 2006; 139: 991–997.

Kim MH, Kim MO, Kim YH, Kim JS, Han HJ. Linoleic acid induces mouse embryonic stem cell proliferation via Ca21/PKC, PI3K/Akt, and MAPKs. *Cell Physiol Biochem* 2009;23:53–64.

Lee MY, Ryu JM, Lee SH, Park JH, Han HJ. Lipid rafts play an important role for maintenance of embryonic stem cell self-renewal. *J Lipid Res* 2010; 51:2082–2089.

Lorimore SA, Coates PJ, Wright EG. Radiation-induced genomic instability and bystander effects: inter-related nontargeted effects of

exposure to ionizing radiation. *Oncogene*. 2003; 13;22(45):7058–7069.

Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood*. 1993 1; 81(11):2844–2853.

Verny ME, Hardman WE, Sollars VE. Omega 3 fatty acids reduce myeloid progenitor cell frequency in the bone marrow of mice and promote progenitor cell differentiation, *Lipids in Health and Disease*; 2009; 8:9.

Shabrani NC, Khan NF, Kale VP, Limaye LS. Polyunsaturated fatty acids confer cryoresistance on megakaryocytes generated from cord blood and also enhance megakaryocyte production from cryopreserved cord blood cells. *Cytotherapy*. 2012;14(3):366–380.

Shannon J, King IB, Moshofskky R, Lampe JW, Gao DL, Ray RM, Thomas DB. Erythrocyte fatty acids and breast cancer risk: a case-control study in Shanghai, China, *Am J Clin Nutr*; 2007; 85(4): 1090–1097.

Shen Y, Nilsson SK. Bone, microenvironment and hematopoiesis. *Curr Opin Hematol.* 2012; 19(4):250–255.

Siddiqui NF, Shabrani NC, Kale VP, Limaye LS. Enhanced generation of megakaryocytes from umbilical cord blood-derived CD34(+) cells expanded in the presence of two nutraceuticals, docosahexanoic acid and arachidonic acid, as supplements to the cytokine-containing medium. *Cytotherapy*; 2011;13(1):114–128.

Simopoulos AP. Omega-6/omega-3 essential fatty acids: biological effects, *World Rev Nutr Diet*; 2009; 99: 1–16.

Simopouls AP. The importance of the ratio of omega 3/ omega 6 essential fatty acids, *Biomed Pharmacother*; 2002; 56(8): 365–379.

Thangavelu G, Colazo MG, Ambrose DJ, Oba M, Okine EK, Dyck MK. Diets enriched in unsaturated fatty acids enhance early embryonic development in lactating Holstein cows. *Theriogenology* 2007;68:949–957.

Till JE, McCulloch EA. Repair processes in irradiated mouse hematopoietic tissue. *Ann N Y Acad Sci.* 1964;114:115–125.

Turk HF, Chapkin RS. Membrane lipid raft organization is uniquely modified by n-3 polyunsaturated fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 2013;88:43–47.

Uchida N, Weissman IL. Searching for hematopoietic stem cells: evidence that Thy-1.1 lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy1.1 bone marrow. *J. Exp. Med.* 1992; 175:175–184.

Umegaki K, Uramoto H, Suzuki J, Esashi T. Feeding mice palm carotene prevents DNA damage in bone marrow and reduction of peripheral leukocyte counts, and enhances survival following X-ray irradiation. *Carcinogenesis.* 1997;18(10):1943–1947.

Weiss JF, Landauer MR. Radioprotection by antioxidants. *Ann N Y Acad Sci*. 2000;899:44–60.

Xia S, Li XP, Cheng L, Han MT, Zhang MM, Shao QX, Xu HX, Qi L. Fish oil-rich diet promotes hematopoiesis and alters hematopoietic niche. *Endocrinology.* 2015; 156(8):2821-2830.

Review

BRJ
Biomedical Research Journal

Mathematical Modeling of Viral Epidemics: A Review

Pratip Shil

National Institute of Virology, 130/1Sus Road, Pashan, Pune – 411021, India

Mathematical models to describe transmission and propagation of diseases have gained momentum over the last hundred years. Formulated mathematical models are currently applied to understandthe epidemiology of various diseases including viral diseases viz Influenza, SARS, measles, etc. With the emergence of advanced computing tools, designing mathematical models and generating simulations (numerical solutions) have become feasible. There is an enormous scope for using mathematical models in studying epidemiology of viral diseases through transmission dynamics of outbreaks and in evaluating or predicting the effects of interventions and vaccinations. The influenza pandemic of 2009 and the recent Ebola epidemics of 2014-15 have generated renewed interest in mathematical modelling of epidemics. Here we present a review of the various mathematical models and their applications in the study of virus driven epidemics.

INTRODUCTION

Mathematics has made significant inroads in biology and medicine with mathematical theories and models being used to study and understand various processes or phenomenon including transmission dynamics of diseases (Abidor*et al*., 1979; Anderson, 1991; Aronson *et al*., 1975; Ball *et al*., 2010; Beirne, 1975; Bowman *et al*., 2005; Carrillo *et al*., 2010; Chowell *et al*., 2006a; 2006b; Cohen *et al*., 2004; Hodgkin *et al*., 1952; Kermack *et al*., 1927; Krassowska *et al*., 1994; Meena *et al*., 2010; Michaelis *et al*., 1913; Mishra *et al*., 2010; Shil *et al*., 2008; Smith *et al*., 2004; Yousfi *et al*., 2011). The progress of mathematical sciences including geometry, algebra and analyses over the last few centuries has enriched different branches of biological sciences. Simultaneously, conceptual and scientific challenges from biology have enriched mathematics by leading to innovative thought and development of novel approaches to mathematical theories. Several pioneering examples include age structure of stable populations by Euler 1760 AD, correlation coefficient by Pearson 1903 AD, Markov chains and

Key words: Mathematical modelling, epidemics, viruses, influenza, SARS, Ebola, SEIR, SEIAR.
***Corresponding Author:** Pratip Shil, National Institute of Virology, 130/1Sus Road, Pashan, Pune – 411021, India.
Email: shil.p@gov.in

statistics of language by Markov 1906, dynamics of interacting species by Lotka 1925, game theory by Neumann and Morgenstern 1953, diffusion for gene frequencies by Kimura 1994 (Cohen, 2004). The pandemic caused by the novel Influenza A/H1N1 2009 and more recent Ebola epidemic have resulted in a renewed interest in mathematical modelling of epidemics (Chowell *et al*., 2014; Fraser*et al*., 2009; Lewnard *et al*., 2014).

Mathematical theories and models are used to analyze both data and new ideas in epidemiology. The process of scientific progress is to observe a phenomenon, generate a hypothesis and design experiments to test the hypothesis. Experiments in epidemiology are difficult to design, with serious ethical issues. A mathematical model, on the other hand, is a description of a phenomenon or situation based on a hypothesis. The general process involve certain assumptions on disease propagation, formulation of the assumptions in mathematical terms and translation into a mathematical problem. The mathematical problem then becomes the model for the epidemic. The numerical solution of the models can be obtained by computer simulations and the outputcompared with the real data. Also, the real data can be fitted to a model to deduce several parameters (Brauer, 2009).

The first mathematical model in epidemiology was developed to study the variolation against small pox in increasing life expectancy by Bernouli (Brauer, 2009; Bernouli, 1760)**.** The foundation of mathematical epidemiology was laid by the contribution of several biologists and physicians as P. D. Enko, W. H. Hamer, Sir R. A. Ross, A. G. McKendrick and W.O. Kermack. The works of Ross on malaria (Ross, 1911) and Kermack and McKendrick (Kermack *et al*., 1933) are considered as landmarks in the development of mathematical epidemiology. Ross, based on his extensive research on malaria in India, showed that the disease was spread by the mosquitoes and developed a model describing the transmission (Ross, 1911). He predicted from this model that reduction of the mosquito population would effectively control the malaria epidemic in a geographical area. Further, several disease specific modelling studies including measles, gonorrhea, AIDS, leprosy (Allen *et al*., 1990; Anderson, 1991; Castillo–Chavez *et al*., 1989; Gupte *et al*., 2000; Hethcote *et al*., 1984; Meima *et al*., 1999).

The concept of basic reproduction number was developed in the works of

Kermack and McKendrick (Kermack, *et al.* 1933). The authors analysed disease propagation in: i) diseases where the infected person recovers and gets conferred immunity against the causative agent (viral diseases) and ii) diseases with recovery but without conferred immunity against the causative agent (bacterial and sexually transmitted diseases). The basic reproduction number, universally denoted as R_0, defines the average number of secondary infections generated by an average infective introduced into a wholly susceptible population. The greater the R_0, the more intense is the transmission and hence more severe is the epidemic. The concept of R_0 is the central idea in mathematical epidemiology as it is vital for prediction or description of transmission dynamics of any epidemic.

The current literature review is a compilation of various mathematical modelling studies on epidemic spread of air-borne and vector borne viral diseases. The review by Zhang *et al*. (2001) is referred to for plant viral epidemics, as it is not within the scope of the current review.

Models for air-borne diseases

1) Susceptible - Infectious - Recovered (SIR)

The first mathematical model used to describe an influenza epidemic was developed by Kermack and McKendrick, popularly known as Susceptible-Infectious-recovered or SIR model. It assumes the introduction of one infected individual into a population where the members are not previously exposed to the pathogen and are hence all susceptible (S). Each infected individual (I) transmits to susceptible members of the population with a mean transmission rate β. At the end of the infectious period, the individual recovers and is considered as Recovered (R) member of the population. If the mean recovery rate is α, then the mean transmission period in any individual is given by 1/α. Fig. 1 describes schematically the SIR model of disease transmission. The set of differential equations describing the transmission as per the basic SIR model is given by

$$\frac{dS(t)}{dt} = -\beta S(t) I(t)$$

$$\frac{dI(t)}{dt} = \beta S(t) I(t) - \alpha I(t)$$

$$\frac{dR(t)}{dt} = \alpha I(t) \qquad \text{(Eqn. 1.1)}$$

Here, *S(t)* and *I(t)* denote the numbers of individuals in the Susceptible and Infectious states respectively at any time *t*. The rates of change of *S(t)* and *I(t)* with time are denoted by the derivatives *dS(t)/dt* and *dI(t)/dt* respectively. The total

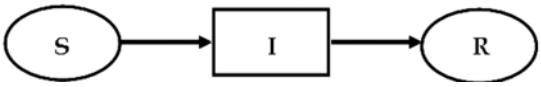


Figure 1. The schematic diagram of the SIR type transmission model. S, I and R denote Susceptible, Infective and Recovered /removed categories of the population.

population is considered constant and is given by $N = S(t) + I(t) + R(t)$, with no one coming in or leaving the system.

The number of susceptible individuals $S(t)$ decreases as the number of incidences (i.e., Infectives $I(t)$) increase. The epidemic peaks then declines as more and more individuals recover and stop transmitting the disease. Considering everyone initially to be susceptible (i.e., at t=0, $S(t) = N$), a newly introduced infected individual can infect on the average $\beta N/\alpha = R_0$individuals. This is the basic reproduction number, R_0. In other words, R_0 describes the average number of secondary infections generated by one infectious individual when introduced into a fully susceptible population. The severity of the epidemic and rates of increase depend on the value of the basic reproduction number. If $R_0 > 1$, then the epidemic will continue. If $R_0 < 1$, then the epidemic will die out. R_0 can be calculated form the growth rate of the epidemic (r) obtained from the cumulative incidences data in the initial growth phase of the outbreak, as:

$$R_0 = \left(1 + \frac{r}{\alpha}\right) \quad \text{(Eqn. 1.2)}$$

The numerical solutions of the ordinary differential equations (Eqn1.1) can be obtained with suitable boundary conditions (appropriate for the disease) using computer simulations. The model has been used to explain the transmission of measles in New York, in 1962 and also repeated outbreaks of the disease between 1930 and 1962 (Anderson, 1991).

The SIR model can be extended to explain occurrence of repeated epidemics in one place due to a pathogen by considering the demographics i.e., addition and removal of individuals from a population through birth and death, respectively. Considering B to be the birth rate per unit time, and a mortality rate (per capita) μ, the Eqn1.1 can be modified as

$$\frac{dS(t)}{dt} = B - \beta S(t) I(t) - \mu S(t)$$

$$\frac{dI(t)}{dt} = \beta S(t) I(t) - \alpha I(t) - \mu I(t) \quad \text{(Eqn. 1.3)}$$

Such modification of the basic SIR model has been used to explain the occurrence of Measles (Anderson 1991). The effects of weather or seasonal variations in human behavior may affect the transmission of a disease. These effects can be incorporated by assuming a transmission rate to be a periodic function in time. A crude

approximation of seasonally forced transmission rate is

$$\beta(t) = \beta_0(1 + A\cos 2\pi t) \quad \text{(Eqn. 1.4)}$$

where, A is the constant defining the amplitude of seasonal variation ($0 \leq A \leq 1$).

The modified SIR models have also been used to explain the dynamics of transmission of various diseases like the measles **(**Allen *et al*., 1990) and influenza (Dushoff *et al*., 2004; Stone, 2007). The SIR model has also been suitably modified to represent or predict spatio-temporal dynamics of disease especially, Influenza outbreak in the erstwhile USSR (Rvachev,1968) and also to incorporate the effects of air travel on influenza pandemics (Baroyan *et al*., 1971; Coburn *et al*., 2009; Rvachev *et al*., 1985).

2) Susceptible - Exposed - Infectious-Recovered (SEIR)

In case of certain infectious diseases, an incubation period or exposed state in an individual following transmission (receiving the causative agent) and till the onset of the symptoms is observed. Hence, the simple SIR model cannot effectively describe transmission of such diseases. Hence, mathematical model should account for the exposed state or the latent state, giving rise to development of the Susceptible- Exposed-Infectious-Recovered or SEIR model.

The SEIR model also assumes introduction of one infected individual into a population where the members are not previously exposed to the pathogen and are hence all susceptible (S). Each individual who received the causative agent (pathogen) exist in the Exposed or Latent state (E) during which he/she is incubating the virus or bacteria but the does not transmit the infection to anyone. With the onset of the symptom, the same individual makes a transition to the Infectious state and is considered as an infected individual (I). If κ be the rate of transition from the Exposed state to the Infectious state, then duration of the mean exposed period or latent phase is $1/\kappa$.Infected individual transmits tosusceptible members of the population with a mean transmission rate β. At the end of the infectious period, the individual recovers and is considered as Recovered (R) member of the population. If the mean recovery rate is α, then the mean transmission period in any individual is given by 1/α. Fig. 2 describes schematically the SEIR model of disease transmission. Considering the constant population size N= S + E + I + R, the set of

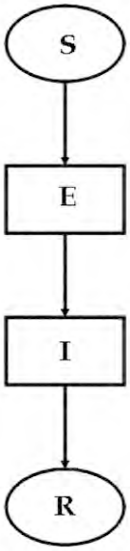


Figure 2. The schematic diagram of the SEIR type transmission model. S, E, I and R denote Susceptible, Exposed (latent), Infective and Recovered /removed categories of the population, respectively.

differential equations describing the transmission as per the basic SEIR model is given by

$$\frac{dS(t)}{dt} = -\beta S(t) I(t)$$

$$\frac{dE(t)}{dt} = \beta S(t) I(t) - \kappa E(t)$$

$$\frac{dI(t)}{dt} = \kappa E(t) - \alpha I(t)$$

$$\frac{dR(t)}{dt} = \alpha I(t) \qquad \text{(Eqn. 2.1)}$$

If we assume that a fraction f of the individuals leaving the infectious state at time *t* recover while the fraction (1-f) die due to disease, then the Eqns. 2.1 can be modified as :

$$\frac{dS(t)}{dt} = -\beta S(t) I(t)$$

$$\frac{dE(t)}{dt} = \beta S(t) I(t) - \kappa E(t)$$

$$\frac{dI(t)}{dt} = \kappa E(t) - \alpha I(t)$$

$$\frac{dN(t)}{dt} = -(1-f)\alpha I(t) \qquad \text{(Eqn. 2.2)}$$

It should be noted that in this case the population is not constant but decreases as more members of the population succumb to the disease. Considering a scenario of no removal by death, the basic reproduction number can be evaluated based on the growth rate of the initial phase of an outbreak for the simple SEIR model as follows.

The growth rate of the epidemic (*r*) can be calculated from the estimates of cumulative number of confirmed infections (y) and the estimated start date and size of the outbreak (t_0 and y_0), respectively, using the equation (Fraser *et al.*, 2009), $y = y_0 e^{r(t-t_0)}$ (Eqn. 2.3)

The basic reproduction number (R_0), is determined using the formula:

$$R_0 = \left(1+\frac{r}{\alpha}\right)\left(1+\frac{r}{\kappa}\right) \qquad \text{(Eqn. 2.4)}$$

with the mean infective period $1/\alpha$ and mean incubation period $1/\kappa$. This gives a more accurate estimation of the R_0compared to the SIR model, where the latent phase was not considered. This is best explained with the help of an example. Gurav *et al*. (2010) has reported about the novel influenza A/H1N1 2009 (Swine flu)

outbreak in a residential school in Panchgani, Maharashtra. Based on the epidemiologic data for the outbreak, Shil *et al*.(2011) derived the intrinsic exponential growth rate (r) to be 0.2341 per day. Assuming the mean incubation period to be 1.5 days and mean infectious period to be 4 days, the R_0 was estimated to be 2.61 (as per Eqn. 2.4). Similar higher values of R_0 and intense transmissions were also observed in various countries for communities with close clustering of people such as village and schools (Guinard *et al*., 2009; Smith *et al*., 2009; WHO, 2009).

The SEIR model with suitable adaptations has been widely used for various diseases including influenza, chicken pox and SARS (Deguen *et al*., 2000; Riley *et al*., 2003). Deguen *et al*. (2000) analysed the seasonal pattern of chicken pox epidemic in France by fitting SEIR model with a periodic contact rate function to weekly chicken pox incidence data collected from 1991-1996. Both the models, assuming either continuous or piecewise constant periodic function, gave reasonable fit to the incidence data and yielded estimates of incubation and infectious periods consistent with the clinically or serologically estimated values. Wang *et al*. (2006) have adapted the SEIR model with a time dependant transmission rate (contact per infectious person per day) for describing the SARS outbreak in Beijing city. The SEIR solution precisely matched the epidemiology data. To study the transmission dynamics of the SARS outbreak in Hong Kong (2003), Small and colleagues (Small and Tse, 2005a; 2005b) adapted the SEIR concept in a 'Small World Model' where transmission was allowed within population clusters and between a random number of geographically distant clusters. Transmission was allowed only between linked nodes/ clusters. This concept could effectively describe the SARS outbreak of 2003 as the computer simulations matched the recorded data.

3) Susceptible - Exposed - Infectious - Asymptomatic - Recovered (SEIAR)

A simple model of disease propagation involving asymptomatic individuals in the population in a scenario without any interventions, that is, an untreated Susceptible - Exposed - Infective-Asymptomatic-Recovered model is explored. In the model the individuals were classified as: Susceptible (*S*) – those who did not have any immunity to the disease; Exposed (*E*) or latent – those

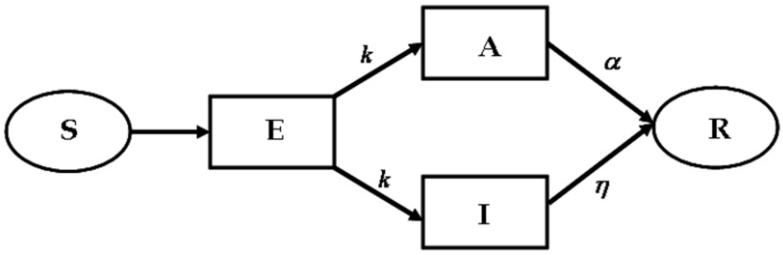


Figure 3. The schematic diagram of the SEIAR type transmission model. S, E, I, A and R denote Susceptible, Exposed (latent), Infective, Asymptomatic and Recovered /removed categories of the population, respectively.

exposed to the virus and incubating it prior to the development of symptoms; 'Infectives' (I) – symptomatic and infectious; Asymptomatic (A) – those testing positive in serological tests/blood tests for the disease, but had no symptoms (were assumed to be partially infectious); and recovered population (R). A flow diagram for the SEIAR model is given in Fig. 3. Following assumptions are made where S, E, I, A, R, denote the numbers of individuals in the Susceptible, Latent (or exposed), Infective, Asymptomatic and Recovered compartments respectively, with the total population size at all times given by $N = S(t) + E(t) + I(t) + A(t) + R(t)$, as: i) Total population at the initial stage was susceptible with no members having immunity through vaccination or any previous exposure. One infective was introduced. ii) There is no transmission from individuals at the Latent (Exposed) state. iii) A fraction p of the latent (E) individuals proceed to Infective (symptomatic) I compartmentat the rate k. The remaining fraction (1-p) goes to the asymptomatic compartment A at the same rate k. iv) The study population is considered constant and no consideration has been made for the addition or removal of individuals. v) Asymptomatic individuals have a reduced capacity to transmit the disease. Let 'q' be the factor that decides reduction in transmissibility of the asymptomatic individuals ($0 < q < 1$) (Poddar *et al*., 2010; Shil *et al*., 2011). vi) Assuming homogeneous mixing within the population, the average member of the population made contact sufficient to transmit infection to βN others per unit time, where β is the transmission rate. vii) A fraction α of the infective individuals and a fraction η of the asymptomatic individuals moved to recovered class per unit time. viii) No restrictions on human behaviour (such as quarantine, wearing of masks) or interventions (as preventive medicine) are imposed.

The transmission process is described by the following set of ordinary differential equations (ODE):

$$\frac{dS}{dt} = -\beta S(I + qA)$$

$$\frac{dE}{dt} = \beta S(I + qA) - kE$$

$$\frac{dI}{dt} = pkE - \alpha I$$

$$\frac{dA}{dt} = (1 - p)kE - \eta A$$

$$\frac{dR}{dt} = \alpha I + \eta A$$

$$\frac{dC}{dt} = \alpha I \qquad \text{(Eqn. 3.1)}$$

Here, *C* denotes the cumulative number of infectives.

Also, all variables are positive at all times ($0 < t < \infty$) (Poddar *et al*., 2010; Shil *et al*., 2011).

The untreated SEIAR model with modifications has been adapted to explain the Influenza A/H3N2 outbreak in Tristan da Cunha 1971 (Mathews *et al*., 2007). Recently we have used this model to explain the transmission dynamics of the Swine flu outbreak at a residential school setting in Panchgani, Maharashtra, India (Shil *et al*., 2011). Analyses of epidemiological data obtained from the outbreak revealed that close clustering within population resulted in high transmissibility with basic reproduction number R_0= 2.61 and transmission rate (β) being 0.001566. The doubling time (the time period in which the size of the outbreak doubles) as calculated from t_d = *ln (2/r)*, where *r* is the exponential growth rate of the epidemic (Shil *et al*., 2011; Wallinga*et al*., 2007), was found to be 2.14 days. The study provided estimates for various parameters for the outbreak such as the partial infectiousness and its duration in the asymptomatic cases. Such parameters were difficult to determine by clinical observations. The study also enabled qualitative assessment of the effect of control measures (behavioural interventions, etc) in controlling the outbreak in a closed population.

4) Complex SEIAR (hospitalization)

We now move on to explore how to incorporate the effects of interventions such as hospitalization into the SEIAR model. Chowell *et al*. (2006) described a complex SEIAR incorporating hospitalization of a fraction of the Infectives. As in the SEIAR model, the members of the population were classified into S, E, I, A, R with J(t) and D(t), in addition denoting the fraction hospitalized and dead respectively, described in Fig. 4.

Initially the entire population is susceptible. It is assumed that an Asymptomatic individual transmits

Figure 1: Genetic poly

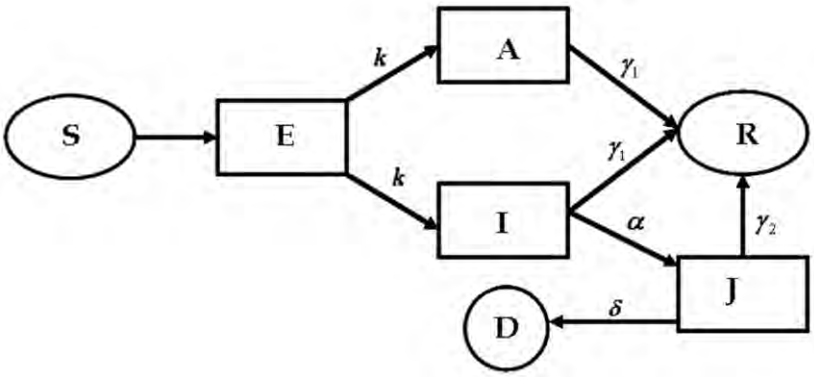


Figure 4. The schematic diagram of the SEIAR type transmission model. S, E, I, A, J, R and D denote susceptible, exposed (latent), infective, asymptomatic, hospitalized (severe cases), recovered and dead categories of the population, respectively.

disease with a reduced transmissibility. Let q ($0 < q < 1$) be the factor that decides the reduction in transmissibility of the Asymptomatics. Susceptible individuals contacting the virus/causative agent move to the latent class at a rate)

$(I(t) + J(t) + qA(t)) / N(t)$,

where β is the transmission rate.

The total population at any time t is given by $N = S(t) + E(t) + I(t) + A(t) + J(t) + R(t)$. Assuming homogeneous mixing of the population and that $J(t)$ are equally infectious as the I(t), the probability of a random contact with the Infective individual is given by,

$(I(t) + J(t) + qA(t) / N(t)$

A fraction ρ of the latent individuals ($0 < \rho < 1$) develop symptoms and become Infective at the rate κ and the rest ($1-\rho$) progress to become asymptomatic A(t) also at the same rate κ. Asymtomatics proceed to recovered R(t) class at the rate γ_1. The infectious individuals are diagnosed and hospitalized at rate α, while some recover with hospitalization at rate γ_2 ordie at the rate δ. The transmission is described by the following set of differential equations:

$$\frac{dS}{dt} = \mu N(t) - \frac{\beta S(t).(I(t) + J(t) + qA(t))}{N} - \mu S(t)$$

$$\frac{dE}{dt} = \frac{\beta S(t).(I(t) + J(t) + qA(t))}{N} - (\kappa + \mu) E(t)$$

$$\frac{dA}{dt} = \kappa(1-\rho)E(t) - (\gamma_1 + \mu) A(t)$$

$$\frac{dI}{dt} = \kappa \rho E(t) - (\alpha + \gamma_1 + \mu) I(t)$$

$$\frac{dJ(t)}{dt} = \alpha I(t) - (\delta + \gamma_2 + \mu) J(t)$$

$$\frac{dR(t)}{dt} = \gamma_1 (A(t) + I(t)) + \gamma_2 J(t) - \mu R(t)$$

$$\frac{dD(t)}{dt} = \delta J(t)$$

$$\frac{dC(t)}{dt} = \alpha I(t) \qquad \text{(Eqn. 4.1)}$$

Here, μ has been considered to be the

rate of birth as well as the rate of natural death in the study population. The cumulative number of confirmed infections is given by $C(t)$. Epidemic data obtained from the Spanish flu pandemic in Geneva was used for fitting to this model and determined the parameters β, γ_1 ,q, α, etc.

The SEIR and SEIAR models had been extended by incorporating various parameters and accounting for public health interventions, behavioral changes or restrictions like school closure, travel restrictions or quarantine, etc in containing spread of viral diseases like influenza (Arino *et al*., 2006; Ballesteros *et al*., 2009; Baroyan *et al*., 1971; Bootsma *et al*., 2007; Chauchemez, 2008; Chowell *et al*., 2006;2007; Coburn *et al*., 2009; Fergussion *et al*., 2006; Longini *et al*., 2005; Mills *et al*., 2004; Sattenspeiel *et al*., 2003;). The effects of vaccination in controlling of the influenza epidemics was also studied (Coburn*et al*., 2009; Galvanic*et al*., 2007; Vardavas *et al*., 2007). The model presented by Longini *et al*. (2005) to describe the influenza (H2N2) pandemic of 1957-58 provided discrete-time simulations based on detailed contact structure. With the advent of the vaccine against novel influenza A/H1N1 (2009), mathematical modelling approach has also been used to decide the effective dosage (Nishiura *et al*., 2009).

Modelling Vector–borne diseases

In case of vector borne diseases transmission depends on several factors including the population of vectors (mosquitoes) and the population of human hosts along with the infected members (within each population) and the nature of vector-host interactions. The first mathematical model for vector borne disease was given by Ross and McDonald. This was improvised upon and adapted for various mosquito borne diseases such as Dengue over the ages (Esteva *et al*., 1999; Kongnuy *et al*., 2011). Described below is a simple model for transmission of mosquito borne disease (Kongnuy *et al*., 2011).

Let us assume that the total populations of both humans and mosquitoes are constants and denoted by H and M, respectively. Let $X(t)$ and $Y(t)$ denote the numbers of infected humans and mosquitoes at any time t, respectively. Let α be the rate of biting on humans by a single mosquito (number of bites per unit time). Then the number of bites on humans per unit time per human is α /H. If b is the proportion of infected bites on humans that produce an infection, the interaction

between the infected mosquitoes $Y(t)$ and the uninfected humans $H - X(t)$ will produce new infected humans of $(\alpha/H)b[H - X(t)]Y(t)$. Let the incubation period in a human be of duration τ_1, then it is possible that some individuals might recover or do not get the disease during this incubation period. Thus, of those individuals infected τ_1 unit times ago, only a proportion

$$(\frac{\alpha}{H})b[H - X(t-\tau_1)Y(t-\tau_1)]\exp(-r\tau_1)$$

is infectious at the present time t, where r is the per capita rate of recovery in humans so that $1/r$ is the duration of the disease in humans. Therefore, the equation for the rate of change in the number of infected humans is

$$\frac{dX}{dt} = -rX(t) + \left(\frac{\alpha}{H}\right)b[H - X(t-\tau_1)]Y(t-\tau_1).\exp(-r\tau_1)$$

(Eqn. 5.1)

Let μ be the per capita rate of mortality in vectors then, $1/\mu$ is the life expectancy of vectors. If the incubation interval of the pathogen in the mosquito has duration τ_2, and c is the transmission efficiency from human to mosquito, then we have the equation for the rate of change in the number of infected mosquitoes as:

$$\frac{dY}{dt} = -\mu Y(t) + \left(\frac{\alpha}{H}\right)c\,X(t-\tau_2)[M - Y(t-\tau_2)].\exp(-\mu\tau_2)$$

(Eqn. 5.2)

If $x(t)$ and $y(t)$ are the proportion of infected humans and mosquitoes at time t, respectively, and m be the number of mosquitoes per human host, then

$$x(t) = \frac{X(t)}{H},$$

$$y(t) = \frac{Y(t)}{M}$$

and

$$m = \frac{M}{H}.$$

Then, we can define the dynamics of the disease by the following set of differential equations:

$$\frac{dx}{dt} = rx(t) + \alpha\, b\, m(1 - x(t-\tau_1))y(t-\tau_2).\exp(-r\tau_1)$$

$$\frac{dy}{dt} = \mu\, y(t) + \alpha\, c\, mx(t-\tau_1)(1 - y(t-\tau_2)).\exp(-\mu\tau_2)$$

(Eqn. 5.3)

The model has been used by Ruan *et al*. (2008) for analyses of malaria and adapted by Massad and coworkers (Massad*et al*., 2010) for description of Dengue transmission. Ruan *et al*. (2008) have estimated the basic reproduction number R_0 by different methods including an adaptation of this model. For a vector borne disease, R_0 may be considered as the number of persons who would be infected from a single person initially infected by a mosquito. According to this model the basic reproduction number is estimated as:

$$R_0 = \frac{\alpha^2 bcm}{r\mu}e^{-r\tau_1}e^{-\mu\tau_2}$$

Considering a primary case with a recovery rate of r, the average time spend in an infectious state is $1/r$. During this

time, since the incubation period in humans has duration τ_1, the average number of mosquito bites received from m susceptible mosquitoes, each with a biting rate α, gives a total of $\frac{\alpha cme^{-r\tau_1}}{r}$ mosquitoes infected by the primary human case. Each of these mosquitoes survives for an average time $1/\mu$ and with another incubation period τ_2 in mosquitoes, makes a total of $\frac{\alpha cme^{-\mu\tau_2}}{\mu}$ infectious bites. The total number of secondary cases is thus estimated to be $\frac{\alpha^2 bcm}{r\mu} e^{-r\tau_1} e^{-\mu\tau_2}$ which is (2). The parameter α appears twice in the expression because the mosquito biting rate controls transmission from humans to mosquitoes and also from mosquitoes to humans.

This model has been used for modelling epidemics driven by arboviral diseases. Massad *et al*. (2010) adapted the model with suitable modifications for estimating the R_0 from Dengue outbreaks of Londrina, and Sao Paulo in Brazil. Based on the simulations that matched the recorded data, the authors concluded that it is possible to have a self-limiting outbreak if $R_0 < 1$ but the vector–human component is greater than 1. Bowman *et al*. (2005) have used similar mathematical modelling and analysis to assess two main anti-West Nile Virus (WNV) preventive strategies, namely: mosquito reduction strategies and personal protection. They proposed a single-season ordinary differential equation model for the transmission dynamics of WNV in a mosquito–bird–human community, with birds as reservoir hosts and culicine mosquitoes as vectors. The public health implication of this is that WNV can be eradicated from the mosquito–bird cycle (and consequently from human population) if the adopted mosquito reduction strategy (or strategies) can make $R_0 < 1$.

Bisanzio *et al*. (2010) explained the transmission of vector borne diseases like Lyme disease and Tick borne Encephalitis using the 'bipartite networks model'. They concluded that aggregation of vectors on hosts have dramatic consequences on epidemic threshold and predicted that the larger networks are able to sustain the epidemic for longer time.

Modelling the transmission of Ebola viral disease (EVD)

The latest major outbreak of Ebola in Guinea, Sierra Leone, and Liberia in 2014 (Barry, 2014) has renewed interest in

modeling of epidemics. Rachah and Torres (2015) defined a simple Susceptible Infectious-Recovered (SIR) mathematical model that describe the 2014 Ebola outbreak in Liberia and validated the same with numerical simulations and available data provided by the World Health Organization. The authors developed a new mathematical model including vaccination of individuals in order to predict the effect of vaccination on the infected individuals over time.

Meltzer *et al*. (2014), used mathematical modeling to estimate and predict number of cases in Ebola outbreaks in Liberia and Sierra Leone. Future predictions based on present available outbreak data helped in estimating the probable scale of outbreak and enabled public health authorities to be prepared for containment and control.

Siettos *et al*. (2015), developed an agent-based model to investigate the epidemic dynamics of Ebola virus disease (EVD) in Liberia and Sierra Leone, 2014. The dynamics of the agent-based simulator evolved on small-world transmission networks of sizes equal to the population of each country, with adjustable densities to account for the effects of public health intervention policies and took into account human behavioral responses to the evolving epidemic.

In a different study, Lewnard *et al*. (2014) developed a transmission model of Ebola virus that was fitted to reported EVD cases and deaths in Montserrado County, Liberia. They used this model to assess the effectiveness of expanding EVD treatment centres, increasing case ascertainment, and allocating protective kits for controlling the outbreak in Montserrado. The estimated value of basic reproductive number for EVD in Montserrado was 2.49 (95% CI 2.38–2.60), and predictions indicated that existing facilities were inadequate to cope with future cases. Their study also revealed importance of protective kits in containing the number of cases. As a public health outcome, these findings prompted authorities to upgrade the facilities.

Modelling Sexually transmitted diseases (STDs)

Mathematical modeling has also been used to describe transmission of sexually transmitted diseases as HIV/AIDS, syphilis, gonorrhoea, etc (Chin *et al*., 1991; Garnett, 1999; 2002; Garnett *et al*., 1997;2000; 80–84). In case of STDs mathematical modelling can describe the

positions of individuals within the network of sexual partnerships allowing identification of risks for acquiring the disease. Since the transmission mechanism for all these diseases are varied considering human behavior and social dynamics, different mathematical modelling was used for the different diseases. For same disease different mathematical approaches have also been described in studies from different countries (Brunham *et al*., 1990; Morris *et al*., 1997; Rapatski *et al*., 2006). A simple model for HIV/AIDS epidemic was described theoretically by Garnett *et al*. (2002), taking into account various parameters for modelling STDs. Considerable work has been carried out on the mathematical analyses of spread of HIV/AIDS (Brunham *et al*., 1990; Morris *et al*., 1997; Rapatski *et al*., 2006), reports on epidemics from India are rare (Rao, 2003). Rao (2003) described different models to explain the transmission patterns of AIDS in India and highlighted that the variable incubation period in patients contribute to complexity in the modelling of AIDS epidemic. Varied social behavior and interaction patterns in human populations across the globe makes it difficult to construct generalized models for STDs.

Advantages and limitations in disease modelling

Study on transmission dynamics of any disease depends on the nature of data and designing of a model that best describes the outbreak scenario. Fitting of epidemiological data helps in optimizing model parameters especially those which cannot be determined by experimentation. For example, the asymptomatic parameters (whether asymptomatics are capable of transmission, how much and for how long, etc) for influenza in humans cannot be estimated by experimentation or observations but can be estimated from modelling studies provided that total number of asymptomatic individuals are known (by serosurvey) for a particular outbreak (Shil, *et al*. 2011). Modelling and simulation studies based on epidemiological data can also help estimate the effectiveness of control measures, and can be employed for evaluation of vaccine efficacy. However, in spite of advantages modelling of epidemics also has limitations.

Limitations in disease modelling results from improper recording of data especially if it involves contact tracing (methods and efficiency may vary country-wise), and /or assumptions for description of the outbreak scenario. This

is true for air-borne diseases. A major limitation of modelling vector borne viral diseases by employing the Ronald Ross model is estimation of the vector data. In any outbreak scenario, estimating the vector population parameters would require detailed survey and sampling of insects (for arboviral diseases) in the affected area and detection of infection in insects using advanced laboratory based techniques, which may not be possible for local medical or municipal authorities.

SUMMARY AND CONCLUSIONS

The review highlights mathematical modeling as an extremely useful tool for study of the transmission dynamics of a wide range of viral diseases such as Influenza, Ebola, SARS, Dengue, WNV, TBE, AIDS, etc. Modeling studies have provided valuable information related to the spread of epidemics and identification of novel interventions for controlling outbreaks. Besides, the models have proved useful in assessing the potential of preventive measures such as mass vaccination, effects of quarantine and hospitalization in controlling the epidemics. However, mathematical models are not always free from approximations because of non-availability of values of some parameters arising from limitations of primary data collection or some proposed parameter/ factor which cannot be estimated clinically or experimentally. On the other hand, mathematical models, if designed carefully and used for data fitting or simulations, will prove extremely useful as compared to clinical/experimental data, particularly, in epidemic situations. Hence, mathematical modelling has an enormous potential in the study of viral epidemics and framing strategies for containing global pandemics. Effective dialogues and coordination between mathematicians, biologists, epidemiologists and clinicians will pave the way with promising collaborations.

ACKNOWLEDGEMENTS

The author acknowledges Dr. D. T. Mourya, Director, National Institute of Virology for meaningful discussions and encouragement.

REFERENCES

Abidor I, Arakelan V, Chernomordik L, Chimadzhev YA, Pastuschenko V, Tarasevich M. Electrical breakdown of bilayer membranes I. The main experimental facts and qualitative discussions *Bioelectrochem Bioenerg* 1979;6:37–52.

Allen L, Lewis T, Martin CF, Stamp M. A mathematical analysis and simulation of a localized measles epidemic. *Appl. Mathematics Computation* 1990;39(1):61–77.

Amira Rachah, Delfim F.M. Torres. Mathematical modelling, simulation and optimal control of the 2014 Ebola outbreak in West Africa. *Discrete Dynamics in Nature Society.* 2015:842792. http://dx.doi.org/10.1155/2015/842792.

Anderson RM, May RM. Infectious disease of humans: Dynamics and control.. Oxford University Press Oxford-New York-Tokyo. 1991.

Arino J, Brauer F. Simple models for containment of a pandemic. *J R Soc Interface* 2006;3:453–457.

Aronson DG, Weinberger HF. Nonlinear diffusion in population genetics, combustion, and nerve propagation. In: Partial Differential Equations and Related Topics, ed. Goldstein, J. Lecture Notes in Mathematics, 446. pp. 5-49. Springer-Verlag, Berlin. 1975.

ASR Srinivas Rao. Mathematical modelling of AIDS epidemic in India. *Curr Sci* 2003; 84(9):1192–1197.

Ball F, Britton T, Sirl D. Household epidemic models with varying infection response. *J Math Biol* 2011;63(2):309–337.

Ballesteros S, Vergu E, Cazelles B. Influenza A gradual and epochal evolution: insights from simple models. *PLoS ONE* 2009;4(10):e7426.

Baroyan OV, Rvachev LA, Basilevsky UV, Ermakov VV, Frank KD, Rvachev MA, Shashkov VA. Modelling of influenza epidemics for the whole country USSR. *Adv. Appl Probab* 1971;2(3):224–226.

Barry M, Traor´e FA, Sako FB, *et al.,* "Ebola outbreak in Conakry, Guinea: epidemiological, clinical, and outcome features," *M´edecineet Maladies Infectieuses* 2014;44(11–12):491–494.

Beirne B. Biological control attempts by introductions against pest insects in the field in Canada. *Can. Entomol*. 1975;107:225–236.

Bernouli D. Essai d'une nouvelle analyse de la mortalite cause par la petite verole. *Mem Math Phys Acad Roy Sci Paris* 1760;1:1–45.

Bisanzio D, Bertolotti L, et al. Modelling the spread of vector borne diseases on bipartite networks. *PLoS ONE* 2010; 5(11):e13796.

Bootsman MC, Ferguson NM. The effects of public health measures on the 1918 influenza pandemic in US cities. *Proc Natl. Acad. Sci. USA,* 2007;104(18):7588–7593.

Bowman C, Gumel AB, van den Driessch P., J. Wu, Zhu H. A mathematical model for assessing control strategies against West Nile virus. *Bulletin of Mathematical Biol.* 2005;67:1107–1133.

Brauer F. Mathematical epidemiology is not an oxymoron. *BMC Public Health* 2009;9(Suppl I):S2.

Brunham RC, Plummer FA. A general model of sexually transmitted diseases and its implication for control. *Med Clin North Am* 1990;74:1339–13352.

Carrillo JA, Cordier S, Mancini S.A decision-making Fokker–Planck model in computational neuroscience. *J Math Biol* 2010; advanced e-pub: doi: 10.1007/s00285-010-0391-3.

Castillo–Chavez C, Ed. Mathematical and Statistical approaches to AIDS epidemiology. *Lect Notes in Biomath* Vol 83, Springer-Verlag, Berlin-heidelberg- New york. 1989;

Chauchemez S, Valleron AJ, Boelle PY, Flahault A, Fergussion NM. Estimating the impact of school closure on influenza transmission from Sentinel data. *Nature* 2008;452:750–754.

Chin J, Iwanga SK. Estimation and projection of adult AIDS cases: a simple epidemiological model. *Bull World Health Organ*. 1991;69:399–406.

Chowell G, Ammon CE, Hengartner NW, Hyman JM. Estimation of the Reproductive number of the Spanish Flu Epidemic in Geneva, Switzerland. Proceedings of the Second European Influenza Conference (St-Julians, Malta, September, 2006)

Chowell G, Ammon CE, Hengartner NW, Hyman JM. Transmission dynamics of the great influenza pandemic of 1918 in Geneva, Switzerland: Assessing the effects of hypothetical interventions. *J Theor Biol* 2006;241(2):193–204.

Chowell G, Nishiura H. Transmission dynamics and control of Ebola virus disease (EVD): a review. *BMC Medicine* 2014,12:196.

Chowell G, Miller MA, Viboud C. Seasonal influenza in the United States, France, and Australia: transmission and prospects for control. *Epidemiol Infect* 2007;136:852–864.

Coburn B, Wagner BG, Blower S. Modeling influenza epidemics and pandemics: insights into the future of swine flu (H1N1). *BMC Medicine* 2009; 7:30.

Cohen JE. Mathematics is biology's next microscope, only better; biology ismathematics' next physics, only better. *PLoS Biology* 2004;2(12):e439.

Deguen S, Thomas G, Chau NP. Estimation of the contact rate in a seasonal SEIR model: application to chickenpox incidence in France. *Statist. Med.* 2000;19:1207–1216

Dushoff J, Plotkin JB, Levin SA, Earn DJ. Dynamical resonance can account for seasonality of influenza epidemics. *PNAS* 2004;101:16915.

En'ko PD. On the course of epidemics of some infectious diseases. *Vrach St Petersberg*, 1889;1008–1010.

Esteva L, Vargas C. A model for dengue disease with variable human population. *J. Math. Biol.* 1999; 38: 220–240.

Fergussion NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies for mitigating an influenzapandemic. *Nature* 2006; 442:448–452.

Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, *et al.* Pandemic potential of a strain of influenza A (H1N1): Early Findings. *Science.* 2009; 324(5934):1557–1561.

Galvanic AP, Reluga TC, Chapman GB. Long-standing influenza vaccination policy is in accord with individual self interest but not with utilitarian optimum. *Proc Natl Acad Sci* USA 2007;104:5690–5697.

Garnett GP, Aral SO, Hoyle DV, Cates W Jr, Anderson RM. The natural history of syphilis: its implications for the transmission dynamics and control of infection. *Sex Transm Dis* 1997;24:185–200.

Garnett GP. The transmission dynamics of gonorrhea: modelling the reported behaviour of infected patients from Nework, New Jercy. *Philosophical Transactions Roy. Soc London B* 1999;354:787–797.

Garnett GP, Bowden FJ. The epidemiology of bacterial sexual transmitted disease: the problems and opportunities of a cure. *Sex.*

Transm Dis 2000; 27: 588–599.

Garnett GP. An introduction to mathematical models in sexually transmitted disease epidemiology. *Sex. Transm Inf* 2002; 78:7–12.

Guinard A, Grout L, Durand C, Schwoebel V. Outbreak of influenza A (H1N1)v without travel history in a school in the Toulouse District, France, June 2009. *Euro Surveill*. 2009; 14(27):pii=19265.

Gupte MD, Kumar BK, Elangovan A, Arokiasamy J. Modelling epidemiology of leprosy. *Ind. J Leprosy*, 2000, 72(3): 305–316.

Gurav YK, Pawar SD, Chadha MS, Potdar VA, Deshpande AS, Koratkar *et al*. Pandemic influenza A(H1N1) 2009 outbreak in a residential school in Panchgani, Maharashtra, India. *Indian J Med Res* 2010; 132: 67–71.

Hamer WH. Epidemic diseases in England – the evidence of variability and persistency of types. *The Lancet* 1906; 167(4306): 655–662.

Hethcote HW, Yorke JA. Gonorrhea transmission dynamics and Control. *Lect Notes in Biomath* Vol 56, Springer-Verlag, Berlin-heidelberg, New York. 1984.

Hodgkin A, Huxley A. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol*. 1952;117:500–544.

Joshua V, Gupte MD, Bhagavandas M**.** A Bayesian approach to study the space time variation of leprosy in an endemic area of Tamil Nadu, South India..*International Journal of Health Geographics* 2008;7:40.

Kermack WO, McKendrick AG. Contributions to the mathematical theory of epidemics, part I. Proc Roy Soc London 1927;115:700–721.

Kermack WO, McKendrick AG. Contributions to the mathematical theory of epidemics, part II. Proc Roy Soc London 1932;138:55–83.

Kermack WO, McKendrick AG. Contributions to the mathematical theory of epidemics, part III. Proc Roy Soc London 1933;141:94–112.

Kongnuy R, Pongsumpun P. Mathematical modelling for Dengue transmission with the effect of season. *Int. J Biological Life Sci* 2011;7(3):143–147.

Krassowska W, Neu JC. Response of a single cell to an external electric field. *Biophys. J.* 1994;66:1766–1776.

Lewnard JA, Ndeff o Mbah ML, Alfaro-Murillo JA, Altice F L, Bawo L, Nyenswah TG, Galvani AP. Dynamics and control of Ebola virus transmission in Montserrado, Liberia: a mathematical modelling analysis. *Lancet Infect Dis* 2014;14:1189–1189.

Lewnard JA, NdeffoMbah ML, Alfaro-Murillo JA, Altice FL Bawo L, Nyenswah TG, Galvani AP. Dynamics and control of Ebola virus transmission in Montserrado, Liberia: a mathematical modelling analysis. *Lancet Infect Dis.* 2014;14(12):1189–1195.

Longini IM, Nizam A, Xu S, Ungchusak K, HAnshaoworakul W, Cummings DA. Halloran ME. Containing Pandemic influenza at the source. *Science* 2005;309(5737):1083–1087.

Massad E, Coutinho FAB, Burattini MN, Amaku M. Estimation of R_0 from the initial phase of an outbreak of a vector borne infection. *E. Tropical Medicine Intl Health*. 2010;15(1):120–126.

Mathews JD, McCaw CT, McVernon J, McBryde ES, McCaw JM. A biological model for influenza transmission: pandemic planning implications of asymptomatic infection and immunity. *PLoS ONE* 2007;2(11):e1220.

Meena A, Eswari A, Rajendran L. Mathematicalmodelling of enzyme kinetics reaction mechanisms and analytical solutions of non-linear reaction equations. *J Mathematical Chem.* 2010;48(2):179–186.

Meima A, Gupte MD, van Oortmarssen GJ, Habbema JDF. SIMLEP: A simulation model for leprosy transmission and control. *International Journal of Leprosy and Other Mycobacterial Diseases* 1999;67:215–236**.**

Meltzer MI, Atkins CY, Santibanez S, Knust B, Petersen BW, Ervin ED, Nichol ST, Damon IK, Washington ML. Estimating the future number of cases in the Ebola epidemic — Liberia and Sierra Leone, 2014–2015. WHO MMWR 2014; 63(3).

Michaelis L, Menten ML. Kinetics of invertase action. *Biochem Z* 1913;49:333–369.

Mills CE, Robins JM, Lipsitch M. Transmissibility of 1918 pandemic influenza. *Nature* 2004; 432:904–906.

Mishra AC, Chadha MS, Choudhary ML, Potdar VA. Pandemic Influenza (H1N1) 2009 Is Associated with Severe Disease in India. *PLoS ONE* 2010;5(5):e10540.

Morris M, Kretzschmar M. Concurrent partnerships and spread of HIV. AIDS 1997;11:641–648.

Nasell I. Hybrid models of tropical Infections. Springer-Verlag, Berlin-heidelberg- New york. 1992.

Nishiura H, Iwata K. A simple mathematical approach to deciding the dosage of vaccine against pandemic H1N1 influenza. *Euro Surveill*. 2009;14(45):pii=19396.

Poddar V, Chadha MS, Jadhav SM, Mallik J, Cherian S, Mishra AC. Genetic characterization of the influenza A pandemic (H1N1) 2009 virus isolates from India. *PLoS ONE* 2010; 5(3):e9693.

Rapatski B, Klepac P, Dueck S, Liu M, Weiss LI. Mathematical epidemiology of HIV/ AIDS in Cuba during the period 1986-2000. *Mathematical Biosciences and Engg*. 2006;3(3):545–556.

Riley S, Fraser C, Donnelly CA, et al. Transmission dynamics of the etiological agent of SARS in Hong Kong: impact of public health interventions. *Science* 2003;300:1961–1966.

Ross R. The prevention of malaria. 2nd edition, John Murray, London: 1911.

Ruan S, Xiao D, Beier JC. On the Delayed Ross–Macdonald Model for Malaria Transmission. *Bull Math Biol.* 2008;70(4):1098–1114.

Rvachev LA. Modelling experiment of a large-scale epidemic by means of a computer. *Trans USSR Acad. SCiSer Mathematics and Physics* 1968;180(2):294–296.

Rvachev LA, Longini LM. A mathematical model of the global spread of influenza, *Math. Biosci* 1985;*75:3–22.*

Sattenspeiel L, Herring DA. Simulating the effects of quarantine on the spread of 1918 flu in central Canada. *Bull Math Biol* 2003;65:1–26.

Shil P, Bidaye S, Vidyasagar PB. Analyzing the effects of surface distribution of pores in cell electroporation for a cell membrane containing cholesterol. *J Phys D: Appl Phys* 2008;41:551–557.

Shil P, Gurav YK, Chadha MS, Mishra AC. Transmission dynamics of novel influenza A/H1N1 2009 outbreak in a residential school in India. *Curr Sci* 2011;100(8):1177.

Siettos C, Anastassopoulou C, Russo L, Grigoras C, Mylonakis E. Modeling the 2014 Ebola

Virus Epidemic – Agent-Based Simulations, Temporal Analysis and Future Predictions for Liberia and Sierra Leone. *PLoS Curr Outbreaks* 2015;Edition 1. doi: 10.1371/currents.outbreaks.8d5984114855fc425e699e1a18cdc6c9.

Small M, Tse C. Clustering model for transmission of the SARS virus: application to epidemic control and risk assessment. *Physica A* 2005;351:499–511.

Small M, Tse C. Small world and scale free model of transmission of SARS. *Intl J Bifurc Chaos* 2005;15(5):1745–1755;

Smith A, Coles S., Johnson S, Saldana L, Ihekweazu C, O'Moore E. An outbreak of influenza A(H1N1)v in a boarding school in south east England, May –June 2009. *Euro Surveill* 2009;14(27):pii=19263.

Smith KC, Neu JC, Krassowska W. Model of creation and evolution of stable macropores for DNA delivery. *Biophys J* 2004;86:2813–2826.

Stone L, Olinky R, Huppert A. Seasonal dynamics of recurrent epidemics. Nature. 2007;446(7135):533–536.

Vardavas R, Breban A, Blower S. Can influenza epidemics be prevented by voluntary vaccination. *PLoS Comp Biol* 2007;3:e85.

Wallinga J, Lipsitch M. How generation intervals shape the relationship between growth rate and reproductive numbers. *Proc R Soc B* 2007;274:599–604.

Wang J, McMichael AJ, Meng B, Becker NG, Han W, Glass K, Wu J, Liu X, Liu J, Li X, Zheng X. Spatial dynamics of an epidemic of severe acute respiratory syndrome in an urban area. *Bull. World Health Organization* 2006;84(12):965–968.

WHO. A practical guide to Harmonizing virological and epidemiological Influenza surveillance. World Health Organization 2009.

Yousfi N, Hattaf K, Tridane A. Modeling the adaptive immune response in HBV infection. *J Math Biol.* 2011;63(5):933–957.

Zhang XS, Holt J. Mathematical models of crossprotection in the epidemiology of plant virus diseases. *Phytopathol* 2001;91(10):924–934.

Review

BRJ
Biomedical Research Journal

Recent Advances in the Treatment of Malaria

Sohal Satish[1], Jayashree Puttur[1], Evans Coutinho[2], Premlata Ambre[2], Santosh R. Nandan[1]*

[1]Ambernath Organics Pvt. Ltd., Creative Industries Premises, Kalina, Santacruz (E), Mumbai – 400098, India.
[2]Bombay College of Pharmacy, Kalina, Santacruz (E), Mumbai – 400098. India.

Malaria is an infectious disease caused by protozoan parasites belonging to the *Plasmodium* species. The disease has been a major cause of mortality and morbidity, especially in populations of African and South-East Asian countries. A well-developed treatment regimen including the artemisinins as a potent antimalarial and other safety preventive measures have played a major role in reducing global burden of malaria over the years. However, recent reports of drug resistance against the artemisinins should be a wakeup call, for the artemisinins have been the mainstay towards the treatment of the disease in recent past. There is a need for newer antimalarials that can be active on more than one stage of the parasite life cycle. These may be complementary to the artemisinins and may also help in keeping a check on the menace of drug resistance. The current review focuses on clinical drug candidates with activity against more than one stages of the malarial parasite life cycle.

INTRODUCTION

Malaria is an ancient disease that has been decimating humans since ages. Malaria kills around 600,000 people each year, mostly children from sub-Saharan Africa. Modern treatment and insect control programs have been implemented in an attempt to control the disease. As a result, the number of malaria cases globally has decreased from an estimated 262 million in 2000 to 214 million in 2015, a decline of 18% whereas the number of malaria deaths has decreased from an estimated 839,000 in 2000 to 438,000 in 2015, a decline of 48%. According to WHO, most deaths in 2015 were in the African Region (90%), followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%). It is estimated that a cumulative 1.2 billion fewer malaria cases and 6.2 million fewer malaria deaths occurred globally between 2001 and 2015 than would have been the case had incidence and mortality rates remained unchanged since 2000 (WHO, 2015a). In the last few years, the cases of malaria have dwindled; as many countries have

Key words: Malaria, erythrocytic stage, pre-erythrocytic stage, proteasome.
***Corresponding Author:** Santosh R. Nandan, Ambernath Organics Pvt. Ltd., Creative Industries Premises, Kalina, Santacruz (E), Mumbai – 400098, India.
Email: santosh.nandan@gmail.com

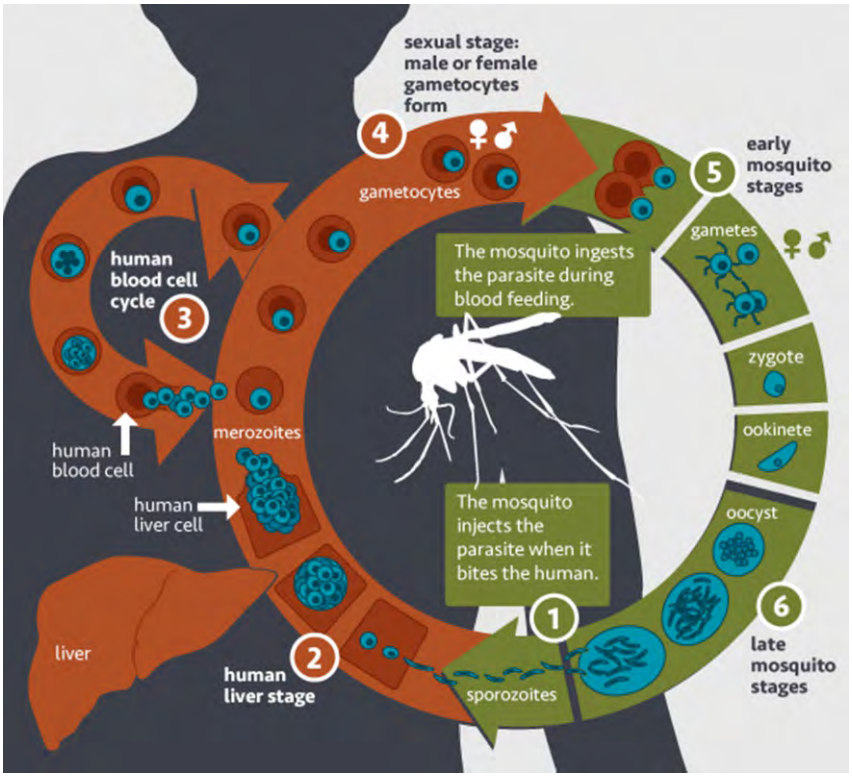


Figure 1: Different stages in the malarial parasite life cycle (NIAID, 2015).

updated their treatment protocol set up by WHO from monotherapy such as chloroquine, amodiaquine to the currently recommended ACT's (Artemisinin-based combination therapy) (WHO, 2015b). However, increasing resistance in *Plasmodium falciparum* and *P. vivax* parasites means current drugs may not remain effective for long.

The disease is most commonly transmitted by an infected female Anopheles mosquito. The parasite has a complicated life cycle; it develops different surface antigens during different stages of its life cycle enabling it to evade immune clearance in the host. The malarial parasite life cycle comprises of 4 stages and every stage has to be considered in order to eradicate the disease. Fig. 1 illustrates the different phases in the parasite life cycle. The mosquito bite introduces the parasites from the mosquito's saliva into a person's blood. The parasites travel to the liver where they mature and reproduce. Five species of *Plasmodium* can infect and be spread by humans. Most deaths are caused by *P. faliparum* because *P. vivax*, *P. ovale* and *P. malariae* generally cause a milder form of malaria. Recently, *P. knowlesi* has also been seen to infect humans, but such cases are rare.

Malarial parasites are continuously evolving and their ability to develop drug resistance forces us to develop newer and more effective drugs. Development of new antimalarials with novel mechanism of action i.e. active against novel targets are needed to fight this war. The idea of developing antimalarials with activity at more than one stage of the life cycle has always been advocated but was not considered practical till a few years ago. A drug candidate acting on both the liver and blood stages or killing the gametes could prove to be a magic bullet in the war against this debilitating disease. Drug research in malaria often focuses on blood stage parasites because they are responsible for the symptoms of the disease and are easier to manipulate in the laboratory. The lack of proper assay for the liver stage has been a major hurdle in developing drugs. The recent advances in phenotypic screening have allowed researchers to target the pre-erythrocytic (liver) stage of the parasite life cycle, which was previously a cumbersome task (Biamonte *et al*., 2013).

MMV (Medicines for Malaria Venture), a non-profit organization based in Geneva, Switzerland aims to develop, discover antimalarials at an affordable cost. MMV works in partnerships with NGO's, research institutions, Pharma companies and is financed with aid from these groups. The R&D portfolio managed by MMV is by far the largest one ever developed for the treatment of malaria (Hentschel and Meguni, 2003). The contribution of MMV in the antimalarial treatment can be easily gauged by looking at the large numbers of preclinical candidates in the global antimalarial drugs portfolio. This review will focus on the latest developments in the treatment of malaria that target more than one stage of the lifecycle of the malarial parasite.

ANTI-MALARIAL TREATMENT

Current Line of Therapy

Widespread resistance to most antimalarial drug classes has led to the global adoption of artemisinin-based combination (ACTs) as first-line therapies. ACT's are a combination of two drugs approved for the treatment of severe malaria. The most popular combinations currently in use are artemether + lumefantrine, artesunate + amodiaquine, artesunate + SP (sulfadoxine + pyrimrethamine) and dihydroartemisinin + piperaquine. The current regimen according to WHO guidelines is a 3-day course of artemisinin which helps in clearing out majority of the parasite with

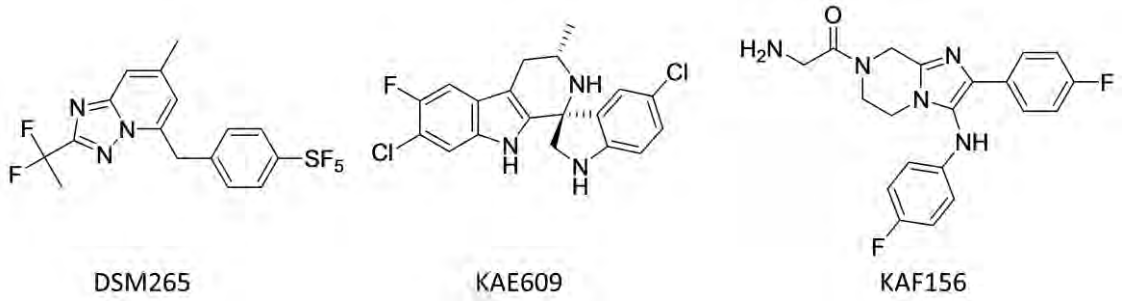


Figure 2: Drug candidates currently in Phase 2 clinical trials.

the remaining parasites are killed by the partner drug (lumefantrine/amodiaquine/ piperaquine) (WHO, 2015b). Artemisinin and its derivatives have rapid onset of action but is quickly cleared from the bloodstream, hence it becomes necessary to combine it with a drug which has a slow clearance rate. Primaquine has the unique distinction of acting on both the liver and blood stage of the malarial parasite. Primaquine, atovoquone and proguanil are used as prophylactics.

Move towards Eradication

Antimalarial drug discovery has always focused on targeting the erythrocytic (blood) stages of the parasite life cycle. The parasite can be easily studied in the blood stage whereas the pre-erythrocytic (liver) stage could be studied only by isolating parasites directly from the mosquito and infecting liver cells for developing an assay (Biamonte *et al*., 2013). The search for drugs acting on the pre-erythrocytic (liver) stage had been stagnant in the past due to lack of proper culture techniques and cumbersome animal models. The development of a phenotypic screening method (Meister *et al*., 2011) by the Novartis-GNF collaboration that targets the parasite lifecycle at the liver stage was a critical advance in the discovery of novel and newer leads. Currently research has focused on developing compounds which are active against both the liver as well as the blood stages of the malarial parasite; such an antimalarial would be extremely effective in eradicating the disease burden in poorer countries.

KAE609 (Fig. 2) is the first antimalarial drug candidate with a novel mechanism of action to achieve positive clinical proof-of-concept in over 20 years. A spirotetrahydro-β-carboline hit was discovered by the phenotypic screening of a Novartis library of 12,000 natural products and synthetic compounds against *P. falciparum*. The spirotetrahydro-β-carboline hit was optimized to improve

potency and oral bioavailability providing the clinical candidate KAE609. In vitro, KAE609 has potent activity against both the pre-erythrocytic (liver) and erythrocytic (blood) stages of the malaria parasite (Novartis, 2014). Spirotetrahydro-β-carbolines inhibit PfATP4, a parasite plasma membrane Na^+-ATPase that regulates sodium and osmotic homeostasis (Yeung *et al*., 2010). A single oral dose of KAE609 provided a cure in a *P. berghei* rodent model of blood-stage malaria. The entire work was carried out at the Novartis Institute for Tropical Diseases in Singapore in collaboration with the Genomics Institute of the Novartis Research Foundation (GNF), the Biomedical Primate Research Centre and the Swiss Tropical Institute. Currently, this compound has completed Phase 2a trials and is undergoing malaria challenge studies in healthy volunteers (controlled human induced blood stage activity) (MMV, 2016).

A Novartis-GNF collaboration identified the imidazolopiperazine scaffold as an attractive hit based on a screening program using a cell based proliferation assay (Nagle *et al*., 2012; Wells *et al*., 2015). Further optimization of these imidazolopiperazine scaffolds led to GNF19 and GNF156 (Fig. 2), of which GNF156 was found to be more promising (Nagle *et al*., 2012). KAF156 (GNF156) not only attacks the asexual but also the sexual stages of malarial parasite life cycle. The compound is currently undergoing Phase 2a clinical trials (MMV, 2016).

DSM265 is a triazolopyrimidine-based inhibitor of the enzyme dihydroorotate dehydrogenase (DHODH) (Phillips *et al*., 2015). It is the first DHODH inhibitor to reach clinical development for treatment of malaria. The compound was found to attack Plasmodium's ability to synthesize the nucleotide precursors required for the synthesis of DNA and RNA. DSM265 (Fig. 2), is a long-acting inhibitor for the treatment and prevention of malaria and which kills *P. falciparum* in blood and liver. DSM265 is a potential drug combination partner for either single-dose malaria treatment or once weekly doses for ongoing disease prevention (Coteron *et al*., 2011). Currently, the compound is undergoing Phase 2 clinical trials in patients affected with *P. falciparum* or *P. vivax* and is in Phase 1b tests where its efficacy against blood stage parasites in combination with OZ439 is undergoing trials (MMV, 2016).

Researchers from University of South

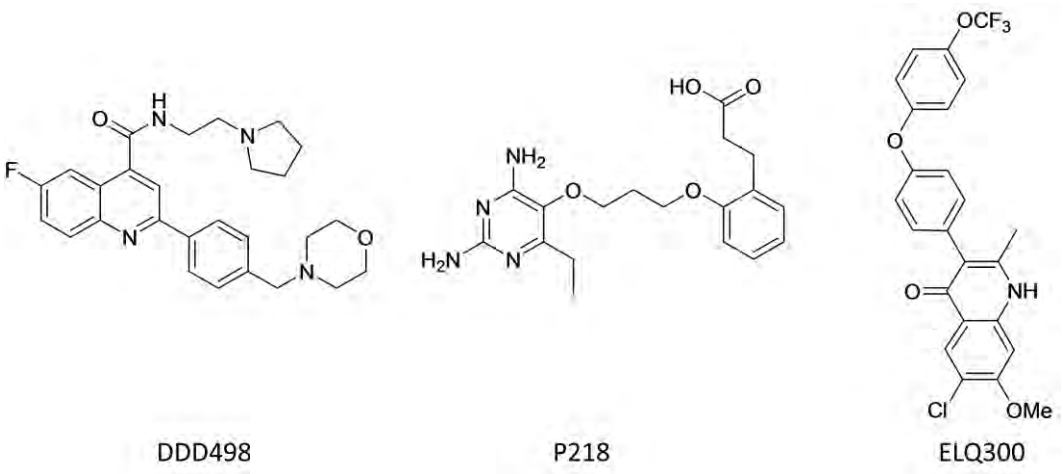


Figure 3: Compounds currently in preclinical stages.

Florida, Drexel University, Monash University, the Portland Veteran Affairs Medical Center, and the Oregon Health and Science University along with Medicines for Malaria Venture (MMV) have developed a new class of anti-malarials - quinolone-3-diarylethers (Broadwith, 2013). ELQ300 drew its inspiration from endochin and the first antimalarial pyridone based drug developed by GSK. The diaryl ether group, part of the pyridone based compound was found to improve its metabolic stability. ELQ300 (Fig. 3) was selected as a preclinical candidate since it targets the liver and blood stages of falciparum malaria, as well as the forms that are crucial to transmission of the disease namely the gametocytes, zygotes, and ookinetes. ELQ300 inhibits the mitochondrial cytochrome bc_1 complex, responsible for ATP and pyrimidine synthesis. It is believed that it would be difficult for the parasite to develop resistance compared to existing drugs targeting the same pathway (Nilsen *et al*., 2013). However, poor aqueous solubility and high crystallinity proved to be an obstacle in the clinical development of this compound. However, a bioreversible O-linked carbonate ester prodrug of the compound, named ELQ 337 (Miley *et al*., 2015), was found to deliver the active drug at concentrations sufficient for single dose cure.

Dundee University in collaboration with MMV developed DDD498 (Fig. 3), a new drug candidate which demonstrates the potential to address a variety of clinical needs, including single-dose treatment, blocking transmission and chemo-protection. DDD498 was developed from a screening programme against blood-stage malaria parasites. This drug targets

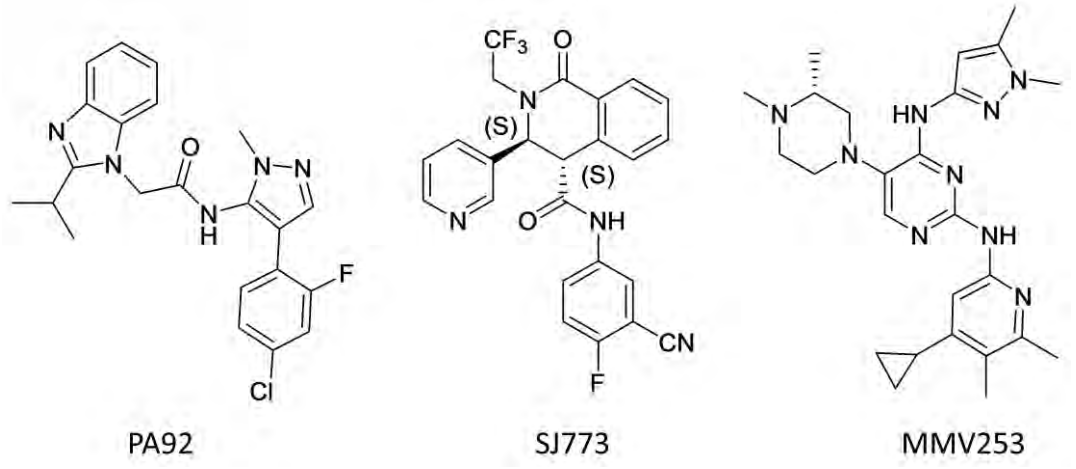


Figure 3.1: Compounds currently in preclinical stages.

the translation elongation factor 2 (eEF2), which is responsible for the GTP-dependent translocation of the ribosome along messenger RNA, and is essential for protein synthesis (Baragana *et al*., 2015). Merck Serono and MMV joined hands to develop this potential antimalarial therapy (MMV, 2015). DDD498 showed an EC_{50} < 1 nM against the liver schizont forms of *P. berghei* and *P. yoelii*. DDD498 potently inhibited both male and female gamete formation at similar concentrations. DDD498 blocked subsequent oocyst development in the mosquito after 7 days with an EC_{50} of 1.8 nM (Baragana *et al*., 2015). This compound is currently undergoing preclinical GLP toxicology studies (MMV, 2016).

BIOTEC (National Center for Genetic Engineering and Biotechnology, Thailand) together with the MMV, developed P218 (Fig. 3) a dihydrofolate reductase inhibitor. Mutations in PfDHFR lead to change in its geometry, thereby restricting the activity of pyrimethamine (Yuthavong *et al*., 2012). Using SBDD, the team designed P218 such that it shows irreversible inhibition. P218 shows excellent selectivity toward PfDHFR, thereby providing safety to humans. The clinical status of this candidate is not known at this time.

Small molecules numbering 500,000 were screened from the AZ (AstraZeneca) collection and TAPs (triamino-pyrimidines) were identified as promising lead series for further evaluation. The compounds have a novel mechanism of action involving inhibition of V-type H^+ ATPase. Medicinal chemistry optimization of TAPs resulted in selection of MMV253 (Fig. 3.1) as a candidate drug with ideal properties like novel chemical class, novel mechanism of action, fast kill

in-vitro and *in vivo*, predicted long half-life in humans and good safety margins in rats and guinea pigs (Hameed *et al*., 2015). TAPs offer the potential for single dose cure in combination with suitable partner drugs as the reported half-life in humans is 36 hours. It is active against multiple strains of *P. falciparum* including those resistant to current antimalarials as well as novel antimalarials in clinical development. The TAPs kill plasmodium parasites rapidly, and the emergence of spontaneous resistance under *in vitro* conditions to this chemical class is rare. The compound is expected to complete preclinical studies soon.

A team of scientists from Drexel University, University of Washington and GNF identified pyrazoleurea and pyrazoleamide derivatives as hits via structure based *in silico* screening of compound libraries. These molecules displayed good activity against both *P. falciparum* and *P. vivax* in animal studies. Optimization of the hits gave rise to 3 lead compounds with nanomolar activity. Of the three, PA92 (Fig. 3.1) was chosen as the drug candidate for further studies. Once inside the host, the parasite induces changes in the host cell membrane so that more nutrients are taken in, which triggers an increase in sodium concentration within red blood cells. The parasite keeps its own sodium levels low with the help of a protein (PfATP4), which pumps sodium out of the parasite. PA92 inhibits this pump causing increase in the Na^+ concentrations within the parasite. This results in excessive water intake, cell swelling and eventually, bursting of the parasite (Vaidya *et al*., 2014).

In search of compounds that inhibit proliferation of parasites, researchers from St. Jude Children's Research hospital in collaboration with MMV and other universities executed a whole-cell phenotypic HTS of more than 1.2 million compounds to identify novel chemicals that kill the malaria parasite (Jimenez-Diaz *et al*., 2014). Three high-priority lead series from this work were pursued: the dihydroisoquinolones (DHIQs), dihydropyridines (DHPs), and diamino-napthoquinones (DANQs). DHIQs was found to be the most promising series, further optimization of the lead led to the development of SJ773 (Fig. 3.1), a fast parasite clearing drug candidate approved for clinical studies by MMV. (+)-SJ733 acts on a cation-transporting ATPase which is responsible for maintaining low intracellular Na^+ levels in the parasite. Treatment of parasitized erythrocytes with (+)-SJ733 in vitro caused a rapid

perturbation of Na^+ homeostasis in the parasite. This disturbance in the level of Na^+ was followed by profound physical changes in the infected cells, including increased membrane rigidity and externalization of phosphatidylserine, consistent with eryptosis (erythrocyte suicide) or senescence (Jimenez-Diaz *et al*., 2014). The mechanism of action of SJ773 and PA92 are similar. Preclinical studies showed this compound as having high oral bioavailability, very good safety margin as well as transmission blocking activity. This compound is currently undergoing preclinical GLP toxicology studies (MMV, 2016).

The proteasome is a multi-component protease complex responsible for regulating key processes such as the cell cycle and antigen presentation (Li *et al*., 2016). Compounds that target the proteasome are potentially valuable tools for the treatment of pathogens that depend on proteasome function for survival and replication. Proteasome inhibitors have been known to inhibit all the stages of the malarial parasite life cycle. However, the major hurdle was lack of selectivity with the parasite over the host cells, making them toxic to humans. Researchers recently have reported a small molecule that can kill the parasite in mice with few side effects. The molecule works by inhibiting the proteasome, the cell's protein-degrading machine, in the parasites but to a much lesser extent in the host. Selective proteasome inhibitors are believed to complement current antimalarial drugs. Also, recent findings suggest proteasome inhibitors suppress artemisinin-resistant strains. Matthew Bogyo and his team at Stanford University School of Medicine first screened a library of peptides to determine sequences favored for degradation by parasite proteasomes but not human ones. They used that information to design selective inhibitors (Goldman, 2016).

They along with the team at the MRC Laboratory of Molecular Biology used cryoelectron microscopy to obtain a structure of the parasite proteasome bound to a designed inhibitor. This structure of the malarial proteasome at the inhibitor-binding site helped further optimization of the inhibitors. A parasite-selective inhibitor, a peptide like molecule called WLL-vs (Fig. 4), was developed that killed artemisinin-sensitive and -resistant malaria parasites. A single dose of WLL-vs substantially reduced parasite levels in mice without any apparent toxic effects. WLL-vs could be combined with artemisinin to decrease the spread of

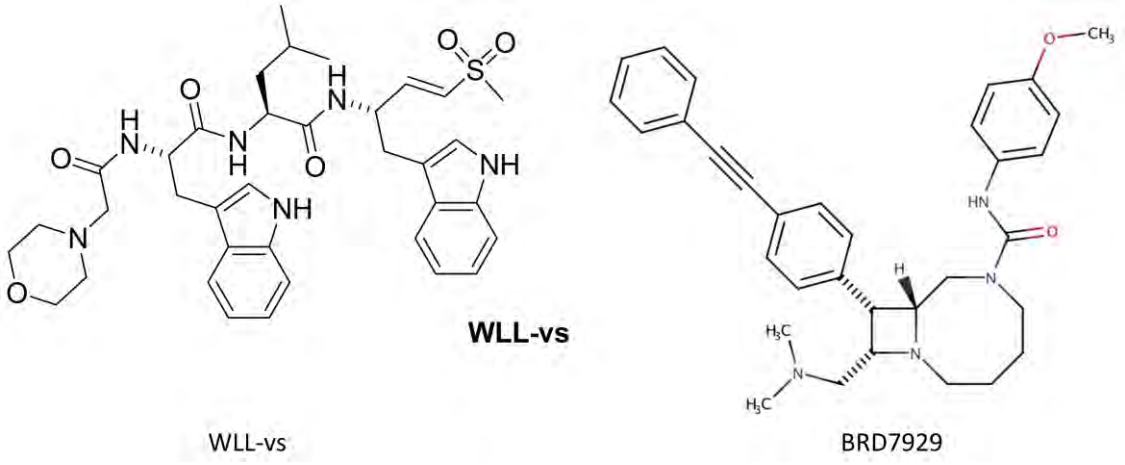


Figure 4: Structures of proteasome inhibitor WLL-vs and bicyclic azetidine BRD7929.

malarial drug resistance, if it can pass efficacy and toxicity trials.

Stuart Schreiber's group at Harvard and Broad Institute (Kato *et al*., 2016) have identified a bicyclic azetidine BRD7929 (Fig. 4) as novel agents that hit all three stages of the malarial lifecycle. They screened a 100,000-member synthetic library built using Diversity Oriented Synthesis that allowed them to access hitherto unknown chemical space. This molecule was capable of blocking transmission and had activity against both the liver and blood stages in multiple in-vivo models (*P. falciparum and P. berghei*). BRD 7929 inhibits the cytosolic Phenylalanyl tRNA synthetase of the parasite thus affecting protein synthesis. BRD 7929 needs further optimization before it can enter the clinic; however, the identification of Phenylalanyl tRNA synthetase as the target should allow researchers around the world to develop newer drugs that act via this mechanism.

FUTURE ASPECTS/CONSIDERATIONS

PfATP4 seems to be the hot target amongst researchers with as many as 3 drug candidates in the clinical trials. All the three drugs have transmission blocking activity in addition with blood stage activity. KAE609 and DDD498 appear to be the most promising of the lot with activity against more than one stage of the parasite life cycle. The current pipeline looks strong and promising with quite a few of them having novel mechanism of action which shows that newer targets have been explored namely eEF2, V type H^+-ATPase. The screening cascade and the hits identified by Stuart Schreiber's group warrants further investigation both in terms of the novel chemical matter and the biological pathways inhibited by them.

The finding of the structure of protein used by mosquito to infect the humans could help in the development of vaccine (Wilson, 2016). The early signs showed by CRISPR and proteasome inhibitors are promising and it is quite hopeful that they would be part of the treatment agenda in the future (Johnson, 2015). MMV has played a major role in the buildup of this pipeline of drugs. MMV's R&D portfolio also includes many drug combinations which are there in the later stages of clinical trials. Though the drugs which are there in the pipeline propose to be one-man army, it would be more logical for these drugs (if approved for human use) to be given in combination with artemisinin derivatives. Investments in R&D and collaboration with various other research organizations have proved to be a winning formula in speeding up the process of drug discovery in the malaria context. One may never know how many compounds synthesized across the world, because of lack of sufficient funding or unavailability of proper techniques/ technologies have seen its way into the bin. It's not surprising to see the amount of contribution of developed countries in R&D activities. So, it becomes imperative that the respective governments take these issues seriously.

A complete ideal package would be a molecule that can target the blood stage of the disease to alleviate the symptoms, the liver stage to prevent relapses, and the transmission stage to protect other humans. Of late researchers are cracking open the doors of genomics to seek an answer to this problem. A malaria vaccine hence is very much a possibility in the near future. Continued progress in combating malaria requires development of newer drugs with broad-ranging activity against all manifestations of the disease. Increased investment in the R&D, more collaborative efforts and disciplined follow ups of the protocols set up by WHO would play a big role towards eradication of malaria. Antimalarial strategies for prevention are ideally a balanced use of mosquito control, anti-Plasmodium treatments, and a general improvement of sanitation and awareness, strategies which the developed countries used to eradicate malaria. Expanding the existing robust pipeline, to create and enlarge the range of combination therapies against blood stage and other parasite stages can go a long way in helping reach the much awaited goal of elimination of malaria.

REFERENCES

Baragana B, Hallyburton I, Lee MCS, Norcross NR, Grimaldi R, Otto TD, *et al*. A novel multiple-stage antimalarial agent that inhibits protein synthesis. *Nature* 2015;522(7556):315–320.

Biamonte MA, Wanner J, Le Roch KG. Recent advances in malaria drug discovery. *Bio Med Chem Lett* 2013;23(10):2829–2843.

Broadwith P. New antimalarial drug class resistance ELQ-300 quinolone. *Chem World* 2013. Available at: http://www.rsc.org/chemistryworld/2013/03/new-antimalarial-drug-class-resistance-elq-300-quinolone (accessed on: 25th June 2016).

Coteron JM, Marco M, Esquivias J, Deng X, White KL, White J, *et al*. Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent *Plasmodium falciparum* dihydroorotate dehydrogenase inhibitors with clinical candidate potential. *J Med Chem* 2011;54(15):5540–5561.

Goldman B. Researchers create compound that combats drug-resistant malaria parasites, spares human cells. *Press Release, Stanform Medicine News Centre* 2016 Available at: https://med.stanford.edu/news/all-news/2016/02/researchers-create-compound-that-combats-malaria.html (accessed on 25th June 2016).

Hameed S, Solapure S, Patil V, Henrich PP, Magistrado PA, Bharath S, *et al*. "Triaminopyrimidine is a fast-killing and long-acting antimalarial clinical candidate. *Nat Comm* 2015;6:6715.

Hentschel C, Itoh M. The resurgence of malaria and the role of the medicines for malaria venture. *Medicines for Malaria Venture Brochure* 2003.

Jimenez-Diaz MB, Ebert D, Salinas Y, Pradhan A, Lehane AM, Myrand-Lapierre ME, *et al*. "(+)-SJ733, a clinical candidate for malaria that acts through ATP4 to induce rapid host-mediated clearance of *Plasmodium*. *Proc Natl Acad Sci USA* 2014;111(50):E5455–E5462.

Johnson GB. Can CRISPR Eliminate Malaria? *Biology Writer* 2015. Available at: http://biologywriter.com/can-crispr-eliminate-malaria/ (accessed on 22nd October 2016)

Kato N, Comer E, Sakata-Kato T, Sharma A, Sharma M, Maetani M, *et al*. Diversity-oriented synthesis yields novel multistage antimalarial inhibitors. *Nature* 2016;538:344–349.

Li H, O'Donoghue AJ, van der Linden WA, Xie SC, Yoo E, Foe IT, *et al*. Structure-and function-based design of *Plasmodium*-selective proteasome inhibitors. *Nature* 2016;530:233–236.

Medicines for Malaria Venture. Interactive R&D portfolio. *MMV* 2016. Available at: http://www.mmv.org/research-development/interactive-rd-portfolio (accessed on 25th June 2016).

Medicines for Malaria Venture. Merck Serono and MMV sign agreement to develop potential antimalarial therapy. *MMV* 2015. Available at: http://www.mmv.org/newsroom/press-releases/merck-serono-and-mmv-sign-agreement-develop-potential-antimalarial-therapy (accessed on: 25th June 2016).

Meister S, Plouffe DM, Kuhen KL, Bonamy GM, Wu T, Barnes SW, *et al*. Imaging of Plasmodium liver stages to drive next-generation antimalarial drug discovery.

Science 2011;334(6061):1372–1377.

Miley GP, Pou S, Winter R, Nilsen A, Li Y, Kelly JX, *et al*. ELQ-300 prodrugs for enhanced delivery and single-dose cure of malaria. *Antimicrob Agents Chemother* 2015;59(9):5555–5560.

Nagle A, Wu T, Kuhen K, Gagaring K, Borboa R, Francek C, *et al*. Imidazolopiperazines: lead optimization of the second-generation antimalarial agents. *J Med Chem* 2012;55(9): 4244–4273.

National Institute of Allergy and Infectious Diseases. Life cycle of the malaria parasite. *NIAID* 2015. Available at: https://www.niaid.nih.gov/lab-sections/3135 (accessed on 25th June 2016).

Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, *et al*. Quinolone-3-diarylethers: a new class of antimalarial drug. *Sci Transl Med* 2013;5(177):177ra37.

Novartis. KAE609 shows promise as next generation treatment for malaria. *Novartis Malaria Initiative* 2014. Available at: http://www.malaria.novartis.com/newsroom/press-releases/2014-07-kae609.shtml (accessed on 25th June 2016).

Phillips MA, Lotharius J, Marsh K, White J, Dayan A, White KL, *et al*. A long-duration dihydroorotate dehydrogenase inhibitor (DSM265) for prevention and treatment of malaria. *Sci Transl Med* 2015; 7(296):296ra111.

Vaidya AB, Morrisey JM, Bergman LW. Pyrazoleamide compounds are potent antimalarials that target Na^+ homeostasis in intraerythrocytic *Plasmodium falciparum*. *Nat Comm* 2014:5:5521.

Wells TNC, van Huijsduijnen RH, Van Voorhis WC. Malaria medicines: a glass half full? *Nat Rev Drug Discov* 2015; 14:424–442.

Wilson A. 3D protein map offers new malaria vaccine hope. Walter+Eliza Hall Institute of Medical Research. Available at: http://www.wehi.edu.au/news/3d-protein-map-offers-new-malaria-vaccine-hope (accessed on 22nd October 2016)

World Health Organization. World Malaria Report 2015a;WHO, Geneva.

World Health Organization. Guidelines for the Treatment of Malaria. 3rd Edn. 2015b;WHO, Geneva.

Yeung BKS, Zou B, Rottmann M, Lakshminarayana SB, Ang SH, Leong SY, *et al*. Spirotetrahydro β-carbolines (spiroindolones): A new class of potent and orally efficacious compounds for the treatment of malaria. *J Med Chem* 2010; 53(14):5155–5164.

Yuthavong Y, Tarnchompoo B, Vilaivan T, Chitnumsub P, Kamchonwongpaisan S, Charman SA, *et al*. Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proc Natl Acad Sci USA* 2012;109(42):16823–16828.

Research Article

BRJ
Biomedical Research Journal

Biomagnetic Interaction of Functionalized Iron Oxide Nanoparticles with Bovine Serum Albumin

Mayank Gupta[1] and Sudeshna Chandra[2]*

[1]Department of Metallurgical Engineering and Materials Science, Indian Institute of Technology Bombay, Powai, Mumbai – 400076, India.
[2]Department of Chemistry, Sunandan Divatia School of Science, SVKM's NMIMS University, Vile Parle (West) Mumbai – 400056, India.

Functionalized iron oxide (magnetic) nanoparticles are promising candidate for detection and sensing of target molecule as they can be manipulated and detected through magnetic interactions. The biological recognition moiety of the functionalized coating results in binding of the target analyte which causes a change in the interaction of the nanoparticles under the influence of an external magnetic field. This forms the basis of the fabrication of a bio-magnetic sensor. The current study reports the use of three different macromolecules viz. glycol chitosan (GC), poly ethylene glycol methyl ether (PEGME) and poly sodium stereo-4 sulphate (PSSNa) to functionalize and cap the magnetic nanoparticles. The magnetic nanoparticles were characterized using FTIR, XRD, TEM and TGA to evaluate their structural and surface properties. TEM showed spherical nanoparticles with mean size of ~11, 12 and 13 nm for GC, PEGME and PSSNa-MNPs respectively. TGA evaluates the weight loss of the modified MNPs and confirms the coating on the surface of the MNPs. Bovine serum albumin (BSA) was immobilized on the functionalized MNPs and detection studies were carried out using AC susceptibility studies on a physical property measurement system. Detection of BSA immobilized MNPs was exhibited at 300 K by the measurement of the imaginary part of the magnetic susceptibility over a frequency range and is based on the changes of dynamic magnetic properties of the MNPs, making use of the Brownian relaxation.

INTRODUCTION

Magnetic nanoparticles (MNPs) are of interest to researchers for applications in magnetic fluids (Chikazumi *et al.,* 1987), catalysis (Lu *et al.,* 2004; Tsang *et al.,* 2004), biotechnology/biomedicine (Gupta and Gupta, 2005), magnetic resonance imaging (Mornet *et al.,* 2006; Li *et al.,* 2005), data storage (Hyeon, 2003), and environmental remediation (Elliott and Zhang, 2001). MNPs can also be manipulated under the influence of an external magnetic field. Of the several MNPs, iron oxides are unique due to their non-toxicity, biocompatibility and injectability, indicating biomedical applications like magnetic resonance

Key words: Magnetic nanoparticles, bio-magnetic sensors, AC susceptibility, macromolecules, Brownian relaxation.
***Corresponding Author:** Sudeshna Chandra, Department of Chemistry, Sunandan Divatia School of Science, SVKM's NMIMS University, Vile Parle (West) Mumbai, 400056, India.
Email: sudeshna.chandra@nmims.edu

imaging (MRI), targeted drug and gene delivery, tissue engineering, cell tracking and magnetic bioseparation (Shubayev *et al.,* 2009). Iron oxide nanoparticles after being loaded with drugs and bioactive agents such as peptides and nucleic acids, form distinct particulate systems that may penetrate cell and tissue barriers. This property enables applications in organ-specific therapeutics and diagnostic modalities (McCarthy *et al.,* 2007).

An unavoidable problem associated with nanosized iron oxide nanoparticles is the intrinsic instability for longer duration, due to the tendency to form aggregates thereby reducing surface energy. Further, the bare metallic nanoparticles are easily oxidized in air resulting in loss of magnetism and dispersibility. Hence, it is important to chemically stabilize the bare magnetic nanoparticles against degradation and agglomeration during or after synthesis, for use in various applications. This can be achieved by grafting/coating the nanoparticles with organic species, like surfactants or polymers, or inorganic materials, such as silica or carbon. The protecting materials serves dual purpose by stabilizing the nanoparticles and by providing functionalities for attachment of various ligands.

The MNPs' ability to be functionalized and the property to respond to an external magnetic field provides a useful tool for sensing and detection of target biomolecules. The biological recognition function of the functionalized MNPs results in binding of the target analyte which causes a change in the interaction of the particle in presence of an external magnetic field. These sensors detect changes in the stray magnetic field of functionalized MNPs upon binding with the target analyte. The magnetic field sensors are based on anisotropic magnetoresistance (Miller *et al.,* 2002), Hall Effect (Besse *et al.,* 2002), or spin valves (Ferreira et al., 2003; Kemp *et al.,* 2003). Alternatively, a superconducting quantum interference device (SQUID) may be used to detect the biological binding activity through relatively slow magnetic Néel relaxation upon immobilization of the biomagnetic particles (Haller *et al.,* 1999). However, this type of sensing does not discriminate different targets of similar biological binding affinity. A new sensing scheme recently devised, makes use of the Brownian relaxation of magnetization of MNPs (Chung *et al.,* 2003). The dominant relaxation mechanism of magnetization of the particle depends on size of the particle.

For particles less than 10 nm, Néel relaxation is the dominant mechanism, whereas for larger particles, Brownian relaxation is dominant.

Study of the AC susceptibility of nanoparticles is performed by plotting the imaginary part of the complex magnetic susceptibility against the frequency. The frequency at which the peak in the imaginary part of the complex magnetic susceptibility is obtained, is characteristic of size of the nanoparticles. By measuring the change in frequency on addition of the target analyte, change in size of the particle is measured and hence the target analyte is detected. Use of an ideal functional agent which binds to a particular target analyte of known size, helps in its detection by overcoming the inherent weakness present in other magnetic field sensors.

The motivation for the study is to utilize the selective bio-affinity of the functional moiety and magnetic properties of MNPs to design a sensor to detect target bio-molecules. The sensor is based on changes of dynamic magnetic properties of the MNPs using the Brownian relaxation.

EXPERIMENTAL

Materials used

Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), sodium hydroxide, glycol chitosan, poly ethylene glycol methyl ether, poly sodium stereo-4-sulfate and bovine serum albumin (BSA) were procured from Sigma Aldrich, India. 100% ethanol solution used for washing precipitates was obtained from Baker Hughes, India. All other chemicals were of analytical grade and were procured from Loba Chemie Pvt. Ltd., India and used as received. Deionized water was used as the solvent.

The capping agents used were glycol chitosan (GC), poly ethylene glycol methyl ether (PEGME) and poly sodium stereo-4-sulfate (PSSNa).

Preparation of Functionalized Iron Oxide Nanoparticles

The magnetic nanoparticles were prepared by the conventional co-precipitation method with a 2:1 molar ratio of Fe^{3+}/Fe^{2+}. 3 g of $FeCl_3.6H_2O$ and 1.05 g of $FeCl_2.4H_2O$ was dissolved in 40 ml of deionised water and was stirred in a five-necked flask under nitrogen atmosphere for 30–45 min at 500–600 rpm until a temperature of 80°C was reached. 5 M NaOH (10 ml) was added dropwise till the solution turned from orange to black. The reaction mixture was then stirred

vigorously at 800–1000 rpm for 1 h. This was repeated three times, once for each capping (functional) agent. To each reaction mixture, 20 ml of capping agent solution (50 mg/ml concentration) was added 30 minutes prior to completion, following which the system was allowed to cool to room temperature. The solutions obtained were washed alternatively with deionised water and ethanol, and supernatants removed by decantation using a permanent magnet to separate the magnetic precipitates. The resultant black powders were dried at 40–50°C in a vacuum oven. The overall reaction was as follows: $Fe^{2+} + 2Fe^{3+} + 8OH^{-} \rightarrow Fe_3O_4 + H_2O$

The obtained MNPs as stabilized by the capping agents are henceforth referred GC-MNPs, PEGME-MNPs and PSSNa-MNPs.

Immobilization of BSA on functionalized MNPs

50 mg of functionalized MNPs and 50 mg BSA were dispersed in 100 mL deionised water and stirred for 5 hours. The suspension was washed with deionised water, three times. The solution was then centrifuged at 10000 rpm for 10–15 min and the supernatant removed by decantation. The resultant black powder was dried at 40–50°C in a vacuum oven. The resultant nanoparticles are named as BSA-GC-MNPs, BSA-PEGME-MNPs, BSA-PSSNa-MNPs.

Characterization Techniques

The phase purity and identification of the MNPs were done by X-ray diffraction (XRD) with PanAnalytical X-Pert diffractometer using a monochromatised X-ray beam with nickel-filtered Cu-Kα radiation at 4°/min scan rate. Fourier transform infrared (FT-IR) spectra were obtained using Jasco, FT-IR 300E spectrometer with a resolution of 4 cm^{-1}. The TEM micrographs were observed by JEOL JEM 2100 for particle size determination. The thermal analysis of the system was carried out by Thermogravimetric analysis (SDT Q 600). Magnetic properties of MNPs were studied using Vibrating Sample Magnetometer Model: 7410, Lake Shore Cryotonics Inc., Ohio, U.S.A.

Magnetic studies of BSA immobilized functional MNPs

Physical Property Measurement System (PPMS) and Magnetic Property Measurement System (MPMS) from Quantum Design was used to study the magnetic behavior of the BSA immobilized MNPs. PPMS was

configured to detect the magnetic moment of the sample material, from which various magnetic parameters like magnetization, magnetic susceptibility were determined. For the MPMS, superconductivity is the critical enabling technology that provides for production of large, stable magnetic fields, and the ability to measure changes in those fields 14 orders of magnitude smaller. Known weight of powder samples were coated in Teflon and were given for testing.

RESULTS AND DISCUSSION

The samples GC-MNPs, PEGME-MNPs and PSSNa-MNPs were synthesized using a co-precipitation reaction. The functionalized MNPs were characterized by FTIR, XRD, TEM and TGA to evaluate their structural and surface properties. Bovine serum albumin (BSA) as exemplary protein was immobilized on the functionalized MNPs to evaluate performance of the MNPs for use as platform for biomagnetic sensing.

The FTIR spectra of GC and GC-MNPs is given in Fig. 1a. The absorption bands for GC were well resolved, whereas those of GC-MNPs were rather broad and few. The CC stretching peaks of the alkyl chains of GC at 1604 cm^{-1} and 1380 cm^{-1} shifted to 1618 cm^{-1} and 1367 cm^{-1},

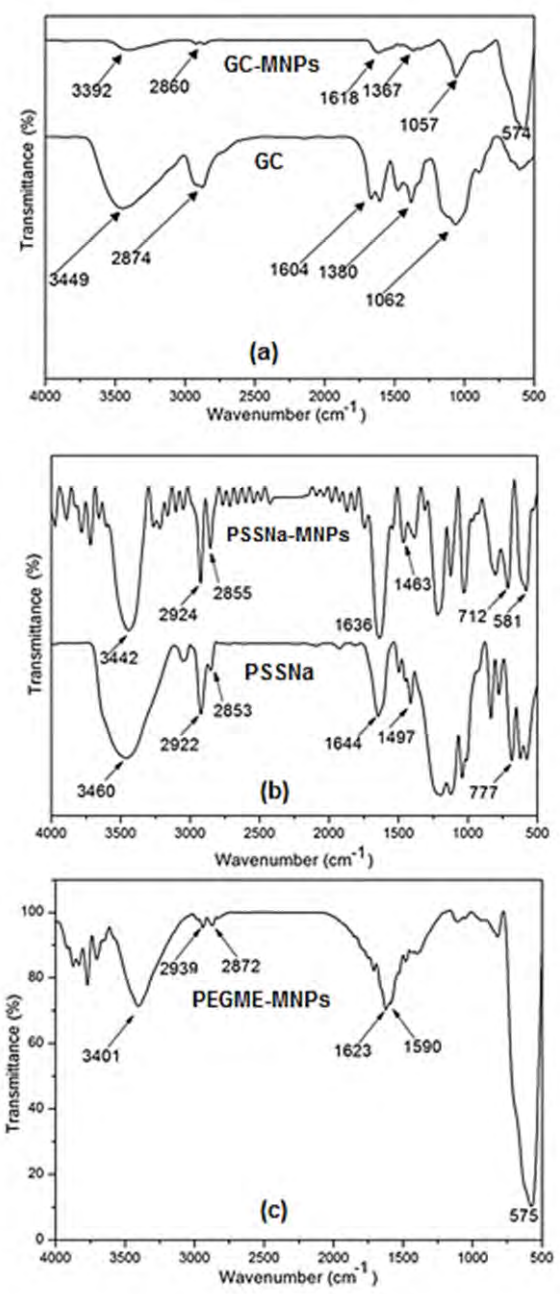


Figure 1. FTIR spectra of (a) GC and GC-MNPs (b) PSSNa and PSSNa-MNPs and (c) PEGME- MNPs.

respectively in GC-MNPs. The peaks at 1062 cm^{-1} and 1057 cm^{-1} are assigned to the CO stretching of the ether bonds. The OH and NH stretching vibrations were observed at 3449 cm^{-1} and 3392 cm^{-1} respectively, while the sharp peaks at 2874

cm^{-1} and 2860 cm^{-1} corresponded to asymmetric and symmetric CH_2 stretching modes. The peak at 3449 cm^{-1} due to NH stretching vibrations appeared broader with a shift at 3392 cm^{-1} in GC-MNPs, indicating that binding of GC to Fe_3O_4 nanoparticles takes place through the amine functionality. Possibly, amine groups of GC form complexes with the Fe-atoms on surface of Fe_3O_4 nanoparticles, weakening the amine bond thereby shifting to lower frequencies.

The FTIR spectra of PSSNa and PSSNa-MNPs is shown in Fig. 1b. The peaks at 1497 cm^{-1} and 1413 cm^{-1} can be assigned to S=O (asymmetric stretching) of the sulfonate bonds. These peaks shift to broad bands at 1463 cm^{-1} and 1387 cm^{-1} in PSSNa-MNPs revealing binding of PSSNa to Fe_3O_4 nanoparticles through sulfonate functionality. The peaks at 2922, 2853, 2924, and 2855 cm^{-1} corresponded to the asymmetric and symmetric CH_2 stretching modes. The peaks at 1644 cm^{-1} and 1636 cm^{-1} are assignable to the CC stretching of benzene ring. The peak at 777 cm^{-1} corresponding to SO stretching of the sulfonate bond in PSSNa shifted to 712 cm^{-1} in PSSNa-MNPs indicating an increase in strength of the bond and suggest bonding of the capping agent to the Fe_3O_4 nanoparticles by sulfonate functionality.

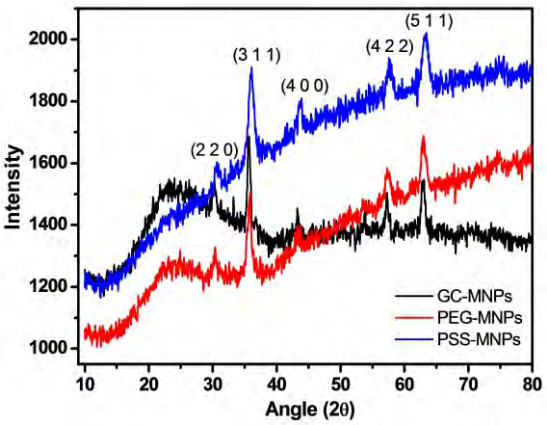


Figure 2. XRD pattern of the functionalized MNPs.

The FTIR spectra of PEGME functionalized MNPs is shown in Fig. 1c. The FTIR analysis of pure PEGME was not possible since PEGME is a waxy material and it could not be powdered along with KBr, for analysis. The peaks obtained at 2939 and 2872 cm^{-1} correspond to the asymmetric and symmetric CH_2 stretching modes (Rufino *et al.,* 2003). The peaks at 1623 cm^{-1} and 1590 cm^{-1} are assigned to the CC stretching of the alkyl chains. The functionalized MNPs showed strong absorption band at ~575 cm^{-1} ascribed to Fe-O stretching vibrational mode of Fe_3O_4 (Ahn *et al.,* 2003).

The XRD pattern of the GC-MNPs, PEGME-MNPs and PSSNa-MNPs (Fig. 2) shows diffraction peaks for planes corresponding to (220), (311), (400), (422), (511) and (440) at 30.4°, 35.5°, 43.2°, 53.8°, 57.3°, 62.7°; 30.4°, 36°, 43.6°, 53.4°, 57.5°, 63.3° and 30.4°, 35.8°, 43.7°, 53.6°, 57.5°, 62.9° 2θ respectively. The data indicates formation of single-phase Fe_3O_4

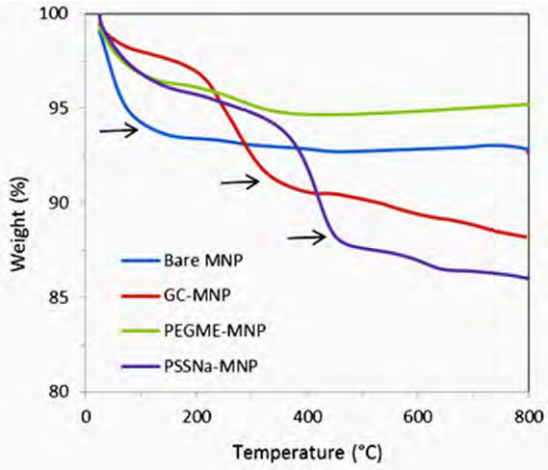


Figure 3. TGA Curves of bare and functionalized MNPs.

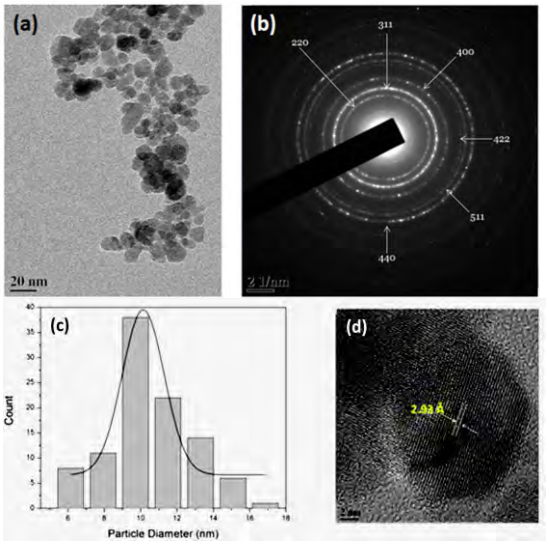


Figure 4. (a)TEM and (b) Electron diffraction pattern (c) particle histogram and (d) HRTEM of GC- MNPs.

inverse spinel structure in the three functionalized MNPs with lattice constants a = 8.37 Å, a = 8.27 Å and a = 8.30 Å respectively, close to reported value of magnetite (JCPDS card No. 88-0315, a = 8.375 Å). The presence of sharp and intense peaks confirms formation of highly crystalline nanoparticles.

The thermogravimetric analysis (TGA) of bare Fe_3O_4, GC-MNPs, PEGME-MNPs and the PSSNa-MNPs are shown in Fig. 3, indicating one weight loss process in Fe_3O_4. The weight loss (~6%) at 100°C is ascribed to the evaporation of adsorbed water molecules.

The functionalized MNPs indicated two weight loss processes, including removal of water below 100°C and an additional weight loss which occurs from 200–400°C assigned to removal of the organic capping agent, as the capping agents burn out at temperatures near 250ºC. At ~550°C, the weight of the sample remained constant and weight loss after this temperature was not observed. It has been observed that the weight loss of bare MNPs are more than the PEGME-MNPs which may be due to delayed combustion brought about by increase in the oxidation temperature. This is caused by their interaction with metal oxide nanoparticles [Karaŏglu *et al*., 2011]. PEG combustion starts at ~340°C and is completely combusted at ~400°C. Further, PEG is not associated with water molecules, hence the weight loss due to water is not observed in contrast to the bare MNPs.

The TEM image of GC-MNPs shows that the particles are spherical although irregular in shape (Fig. 4a). Electron diffraction (Fig. 4b) revealed dense ring

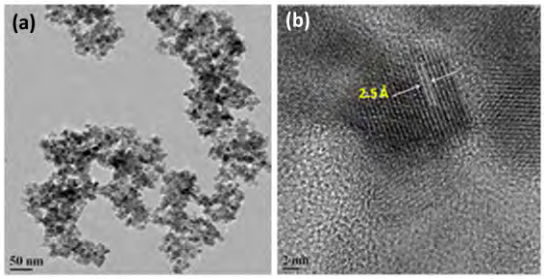


Figure 5. (a) TEM image and (b) HRTEM of PEGME-MNPs.

patterns with d-spacings of 2.94, 2.51, 2.10, 1.70, 1.60, 1.47 Å, matching standard body centered cubic spinel structure (JCPDS card No. 88-0315). The histogram of size distribution of the GC-MNPs (Fig. 4c) showed the mean size of MNPs as 11.41 ± 0.13 nm. The results were similar as with XRD results. Fig. 4d shows the HRTEM image of GC-MNPs. The crystallite in the image has d-spacing of 2.94 Å corresponding to the (220) plane of Fe_3O_4.

The TEM image of PEGME-MNPs also showed the particles as spherical although irregular in shape (Fig. 5a). The mean size of the MNPs is 12.91 nm ± 0.13 nm. Fig. 5b shows the HRTEM image PEGME-MNPs. The crystallite in the image has d-spacing of 2.5 Å corresponding to the (331) plane of Fe_3O_4. In PSSNa-MNPs, HRTEM image shows the crystallite d-spacing is 2.93 Å corresponding to the (220) plane of Fe_3O_4.

Detection studies of BSA immobilized MNPs

To study the immobilization of BSA on the functionalized MNPs, a magnetic sensor scheme based on the changes of dynamic magnetic properties of magnetic nanoparticles suspended in liquids was used. The sensor scheme employed is based on the detection of dynamic magnetic properties (Pankhurst *et al.,* 2003). The nanoparticles were subjected to a small alternating magnetic field with varying frequency. The imaginary part of the magnetic response exhibited by nanoparticles to AC magnetic field with frequency (ω) was recorded. The magnetic response exhibited was expressed by a complex magnetic susceptibility χ.

The imaginary part of the complex magnetic susceptibility (χ′′) corresponds to the out-of-phase response and is expressed as

$$\chi''(\omega) = \frac{\chi_0 \omega\tau}{1 + (\omega\tau)^2}$$

Where χ_0 is the DC magnetic susceptibility and τ is the effective magnetic relaxation time of MNPs.

The value of this imaginary part (χ″) peaks when $\omega = \tau^{-1}$. The effective magnetic relaxation time is proportional to the volume of the MNPs.

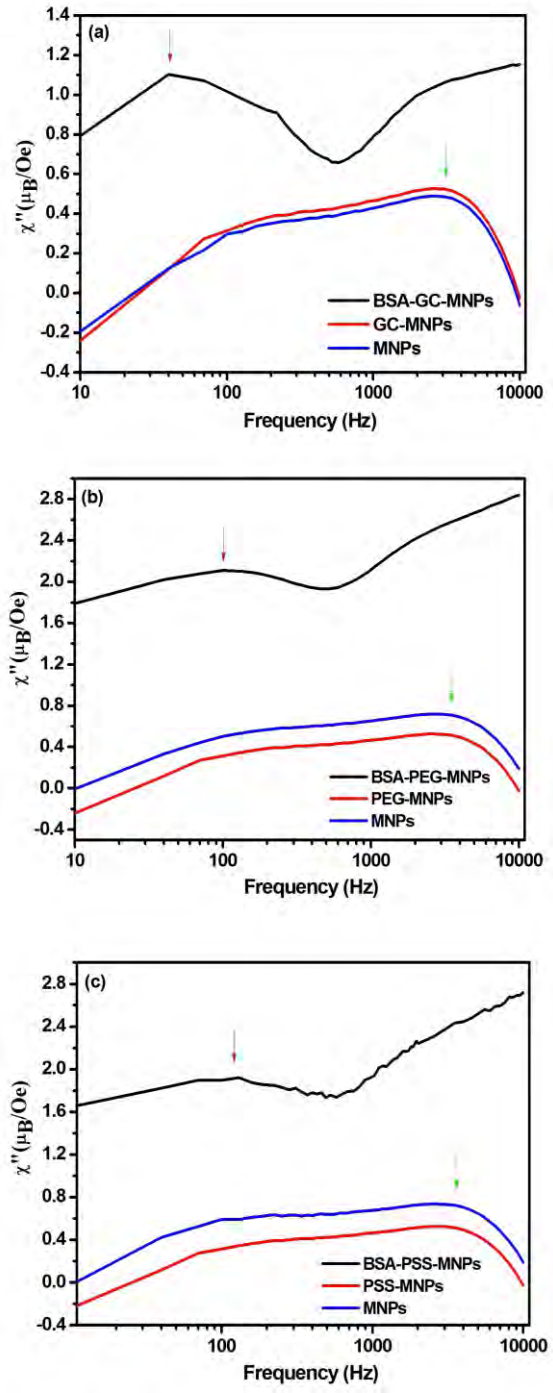


Figure 6. AC susceptibility curves of (a) GC functionalized MNPs (b) PEG functionalized MNPs and (c) PSSNa functionalized MNPs at 300 K at an amplitude of 10 Oe.

PPMS was used to detect immobilization of BSA on the functionalized MNPs by using the above equations. The imaginary part of AC magnetic susceptibility is plotted against frequency. The frequency is varied from 10 Hz to 10,000 Hz while keeping amplitude constant at 10 Oe. These measurements are carried out at two different temperatures viz., 300 K and 10 K. The plot of the imaginary part of the magnetic susceptibility of bare MNPs varies from 0 to 0.25 over the frequency range. The peak value of 0.5 at a frequency of 1250 Hz is shown in Fig. 6. The functionalized MNPs show a very similar parallel plot with a slight offset in values. The offset is a result of a change in the DC magnetic susceptibility of the nanoparticles due to addition of functional agents (Marcon *et al.,* 2012).

At 300 K, decrease in frequency for the peak value of the imaginary part of AC magnetic susceptibility was observed (Fig. 7a-c). The decrease in frequency corresponds to increase in diameter of the functionalized MNPs upon BSA immobilization (Table 1). The increase in diameter corresponds to the size of the BSA molecule, estimated to be 14 nm. An increase in absolute values of AC magnetic susceptibility was observed on addition of BSA. The increase is a result of increase in the DC magnetic susceptibility of the nanoparticles due to immobilization of

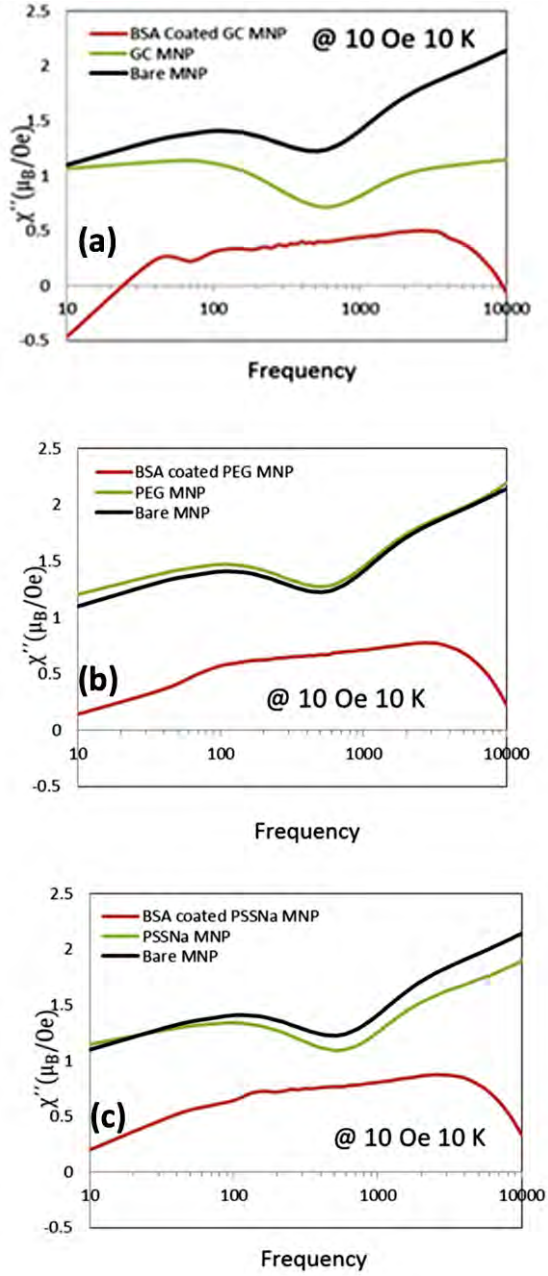


Figure 7. AC susceptibility curves of (a) GC functionalized MNPs (b) PEG functionalized MNPs and (c) PSSNa functionalized MNPs at 10 K and amplitude of 10 Oe.

BSA. DC magnetic susceptibility of a composite particle is a sum of individual DC magnetic susceptibilities of the components.

At 10K, the peak disappeared as shown in Figure 7, due to the fact that 10K is below the freezing point of the liquid. This causes the nanoparticles to be trapped in position in the frozen solution resulting in disappearance of the peak. This also implies that the low frequency peak at room temperature (300K) is due to the rotational diffusive Brownian relaxation of the magnetization.

Table 1. Increase in diameter of functionalized MNPs after immobilization of BSA at 300K and amplitude of 10 Oe.

Functionalized MNPs	Initial Diameter of MNPs (TEM)	Diameter after immobilizing BSA (from AC susceptibility results)	Increase in size
GC – MNPs	11.41 nm	45.28 nm	33.87 nm
PEGME – MNPs	12.91 nm	42.51 nm	29.60 nm
PSSNa – MNPs	13.62 nm	42.02 nm	28.40 nm

CONCLUSIONS

In the current study, magnetic nanoparticles (MNPs) were synthesized and functionalized with macromolecules. The average size of the nanoparticles was below 15 nm. BSA was immobilized on the functionalized MNPs and detection studies were carried out using AC susceptibility studies on a physical property measurement system. Detection of BSA immobilization by functionalized MNPs was exhibited at 300K by the measurement of the imaginary part of the magnetic susceptibility over a frequency range.

ACKNOWLEDGEMENTS

Authors acknowledge the Department of Science and Technology (DST), Govt. of India for providing financial support (Ref. No. SR-WOS-A/CS-45/2010).

CONFLICT OF INTEREST

The authors claim no conflict of interest.

REFERENCES

Ahn Y, Choi EJ, Kim EH, Superparamagnetic relaxation in cobalt ferrite nanoparticles synthesized from hydroxide carbonate precursors. *Rev Adv Mater Sci* 2003;5:477–480.

Besse PA, Boero G, Demierre M, Pott V, Popovic R, Detection of a single magnetic microbead using a miniaturized silicon Hall sensor. *Appl Phys Lett* 2002; 80:4199–4201.

Chikazumi S, Taketomi S, Ukita M, Mizukami M, Miyajima H, Setogawa M, Kurihara Y, Physics of magnetic fluids. *J Magn Mater* 1987;65:245–248.

Chung SH, Hoffmann A, Bader SD, Biological sensors based on Brownian relaxation of magnetic nanoparticles. *Appl Phys Lett* 2003;85:2971–2973.

Elliott DW, Zhang WX, Field assessment of nanoscale bimetallic particles for groundwater treatment. *Environ Sci Technol* 2001; 35:4922–4926.

Ferreira HA, Graham DL, Freitas PP, Cabral JMS, Biodetection using magnetically labeled biomolecules and arrays of spin valve sensors. *J Appl Phys* 2003;93:7281–7286.

Gupta AK, Gupta M, Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* 2005;26:3995–4021.

Haller A, Hartwig S, Matz H, Lange J, Rheinländer T, Kötitz R, Weitschies W, Trahms L, Magnetic relaxation measurement in immunoassay using high-transition-temperature superconducting quantum interference device system. *Supercond Sci Technol* 1999;12:953–955.

Hyeon T, Chemical synthesis of magnetic nanoparticles. *Chem Commun* 2003;32:927–934.

Karaðglu E, Deligöz H, Sözeri H, Baykal A, Toprak MS, *Nano-Micro Lett* 2011;3:25–33.

Kemp JT, Webb C, Davis RW, Sun S, Detection of single micron-sized magnetic bead and magnetic nanoparticles using spin valve sensors for biological applications. *J Appl Phys* 2003;93:7557–7559.

Li Z, Wei L, Gao MY, Lei H, One-Pot Reaction to Synthesize Biocompatible Magnetite Nanoparticles. *Adv Mater* 2005;17:1001–1005.

Lu AH, Schmidt W, Matoussevitch N, Pnnermann HB, Spliethoff B, Tesche B, Bill E, Kiefer W, Schuth F, Nanoengineering of a magnetically separable hydrogenation catalyst. *Angew Chem* 2004;116:4403–4406.

Marcon P, Ostanina K, Overview of Methods for Magnetic Susceptibility Measurement. *PIERS Proceedings* 2012;420–424.

McCarthy JR, Kelly KA, Sun EY, Weissleder R, Targeted delivery of multifunctional magnetic nanoparticles. *Nanomed* 2007;2:153–167.

Miller MM, Prinz GA, Cheng SF, Bounnak S, A model for a magnetoresistance-based biosensor. *Appl Phys Lett* 2002;81:2211–2213.

Mornet S, Vasseur F, Grasset P, Verveka G, Goglio A, Demourgues J, Portier E, Duguet EP, Magnetic nanoparticles design for medical application. *Prog Solid State Chem* 2006;34:237–247.

Pankhurst QA, Connolly J, Jones SK, Dobson J, Applications of magnetic nanoparticles in biomedicine. *J Phys D* 2003;36:167–181.

Rufino ES, Monteiro EEC, Infrared study on methyl metha-crylate-methacrylic acid copolymers and their sodium salts. *Polymer* 2003;44:7189–7198.

Shubayev VI, Pisanic TR, Jin S, Magnetic nanoparticles for theragnostics. *Adv Drug Deliv Rev* 2009;61:467–477.

Takafuji M, Ide S, Ihara H, Xu Z, Preparation of Poly(1-vinylimidazole)-Grafted Magnetic nanoparticles and their application for removal of metal ions. *Chem Mater* 2004;16:1977–1983.

Tsang SC, Caps V, Paraskevas I, Chadwick D, Thompsett D, Magnetically Separable, Carbon-Supported Nanocatalysts for the Manufacture of Fine Chemicals. *Angew Chem Int Ed* 2004;43:5645–5649.

Information for Authors

Biomedical Research Journal
Sunandan Divatia School of Science,
Bhaidas Sabhagriha Building,Bhaktivedanta Swami Marg,
Vile Parle (W), Mumbai - 400056, INDIA.
Email: brj.sos@nmims.edu

Biomedical Research Journal (BRJ) accepts the following article types for publication

Editorial

Authors who are considering submitting an editorial should contact either the Editors-in-Chief with a brief outline of the proposed contribution before submission. Editorials are welcome on any topic; however, they may also be related to articles previously published in the Journal. Editorials have no abstract and no keywords, and are usually restricted to 1000 words, up to 10 references and up to 2 tables or figures. The Editors-in-Chief can be contacted at brj.sos@nmims.edu.

Original Research Articles

Original research articles which have not been published previously, except in a preliminary form may be submitted as original full length research papers. Research articles must contain an abstract, a list of up to six keywords, and are limited to 3,500 words in length.

Review Articles

Review articles which are topical and are a critical assessment of any aspect of mentioned areas. Review articles must contain an abstract, a list of up to ten keywords, and are limited to 5,000 words in length. Authors whose manuscripts exceed 5,000 words are advised to contact the Editorial Office prior to submission.

Letters to the Editor

Letters to the Editor relating to published work in the journal are welcome. Letters should be closely related to the contents of the referred article.

After reading the Instructions to Authors, please visit our online submission system to submit your manuscript.

Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review. Ensure that the following items are present:

- One Author designated as corresponding Author:
  - E-mail address
  - Full postal address
  - Telephone(s) and fax numbers
  - All necessary files have been uploaded
- Keywords (as comprehensive as possible)
- All figure captions
- All tables (including title, description, footnotes)
- The copyright form has been completed and uploaded

Further considerations

- Manuscript has been "spellchecked" and is written in good English
- Title is clear and unambiguous
- If the manuscript is an original research article it should contain a structured abstract, if the manuscript is a review article it should contain an unstructured abstract
- References are in the correct format for this journal
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Web)
- Colour figures are clearly marked as being intended for colour reproduction on the Web (free of charge), and in print or to be reproduced in colour on the Web (free of charge) and in black-and-white in print
- If only colour on the Web is required, black and white versions of the figures are also supplied for printing purposes
- The manuscript conforms to the limits imposed on original research (3,500 words); Review articles (5,000 words), excluding the abstract, keywords, references, tables and figures)

For any further information please contact the Author Support Department at brj.sos@nmims.edu

Prior to Submission

BRJ will consider manuscripts prepared according to the guidelines adopted by the International Committee of Medical Journal Editors ("Uniform requirements for manuscripts submitted to biomedical journals", available as a PDF from (3500 words) http://www.icmje.org). Authors are advised to read these guidelines.

Previous Publication

Submission of an article implies that the work described is:

- Not published previously (except in the form of an abstract or as part of a published lecture or academic thesis)
- Not under consideration for publication elsewhere
- The publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the Publisher.

Ethics

Work on human beings that is submitted to Journal should comply with the principles laid down in the Declaration of Helsinki: Recommendations guiding physicians in biomedical research involving human subjects, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October, 1975, the 35th World Medical Assembly, Venice, Italy, October 1983, and the 41st World Medical Assembly, Hong Kong, September 1989. The manuscript should contain a statement that the work has been approved by the appropriate ethical committees related to the institution(s) in which it was performed and that subjects gave informed consent to the work. Studies involving experiments with animals must state that their care was in accordance with institution guidelines. Patients' and volunteers' names, initials and hospital numbers, should not be used and patient confidentiality must be maintained.

Conflict of Interest

By means of a "Conflict of interest statement", all authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. If there are no conflicts of interest, please state "No conflict of interest declaration". This document should be uploaded as a separate file alongside the submitted manuscript.

Role of the Funding Source

All sources of funding should be declared as an acknowledgment at the end of the text.

Authorship and Acknowledgments

All authors must be accredited on the paper and all must submit a completed Author Form with their submission. The form must be signed by the corresponding author on behalf of all authors and can be scanned and uploaded.

Copyright

Upon acceptance of an article, Authors will be asked to transfer copyright. This transfer will ensure the widest possible dissemination of information. A letter will be sent to the corresponding Author confirming receipt of the manuscript. A form facilitating transfer of copyright will be provided.

If excerpts from other copyrighted works are included, the Author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

General Points

We accept Word format of submitted manuscript. Always keep a backup copy of the electronic file for reference and safety. Save your files using the default extension of the program used. It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. Do not embed "graphically designed" equations or tables, but prepare these using the word processor's facility. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts. Do not import the figures into the text file but, instead, indicate their approximate locations directly in the electronic text and on the manuscript. See also the section on Preparation of electronic illustrations.

To avoid unnecessary errors you are strongly advised to use the "spellchecker" function of your word processor.

Presentation of Manuscript

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Italicize expressions of Latin origin, for example, *in vivo*, *et al*., *per se*. Use decimal points (not commas).

Title Page

Provide the following data on the title page:

Title

Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

Author names and affiliations

Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the Authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the Author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each Author.

Corresponding Author

Clearly indicate who is willing to handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.

Present/permanent address

If an Author has moved since the work described in the article was done, or was visiting at the time, a "Present address"' (or "Permanent address") may be indicated as a footnote to that Author's name. The address at which the Author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Suggestions for reviewers

Please supply the names of up to three potential reviewers for your manuscript. Please do not suggest reviewers from your own institution, previous or current collaborators. Please provide full names, addresses and email addresses of suggested reviewers. Please note: the final choice of reviewers is that of the Editor and the journal reserves the right for choice of final reviewers.

Abstract

A concise and factual abstract of no more than 250 words is required. The abstract must be structured for original research articles. The abstract should be divided by subheadings as follows: Objectives, Materials and Methods, Results, Discussion and Conclusion.

The abstract should not be structured for review articles. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separate from the article, so it must be able to stand alone.

Keywords

After the abstract provide a maximum of six keywords, to be chosen from the Medical Subject Headings from Index Medicus. These keywords will be used for indexing purposes

Abbreviations

Define abbreviations or acronyms that are not standard in this field at their first occurrence in the article; in the abstract and also in the main text after it. Ensure consistency of abbreviations throughout the article.

Text

This should start on the third page and should be subdivided into the following sections: Introduction, Patients or Materials and Methods, Results, Discussion and Conclusions, Acknowledgements.

References

Responsibility for the accuracy of bibliographic citations lies entirely with the authors. Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. "Unpublished data" and "Personal communications" are not allowed. As an alternative, say in the text, for example, '(data not shown)' or '(Dr D. Saranath, School of Science, NMIMS (Deemed-to-be) University, Mumbai)'. Citation of a reference as "in press" implies that the item has been accepted for publication and a copy of the title page of the relevant article must be submitted.

Indicate references by (first author, year) in the text.

Examples:

Kulkarni J, Khanna A. Functional hepatocyte-like cells derived from mouse embryonic stem cells: A novel *in vitro* hepatotoxicity model for drug screening. *Toxicol In Vitro* 2006;20:1014-1022.

Bhatnagar R, Dabholkar J, Saranath D. Genome-wide disease association study in chewing tobacco associated oral cancers. *Oral Oncol* 2012;48(9):831-835.

Molinolo AA, Hewitt S, Amornphimoltham PI, Keelawat S, Saranath D, Gutkind JS *et al*. Dissecting the Akt/mTOR signaling network: emerging results from the head and neck cancer tissue array initiative. *Clin Cancer Res* 2007;13:4964-4973.

Saranath D. Integrated Biology and Molecular Pathology of Oral Cancer. *In*: Saranath D, editor. Contemporary Issues in Oral Cancer. Oxford Press, 2001:30-71.

List all authors if the total number of authors is seven. For more than seven authors, first six authors should be listed, followed by "*et al.*" For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (*J Am Med Assoc* 1997;277:927-934).

Figure Captions, Tables, Figures and Schemes

Present these, in the given order, at the end of the article. They are described in more detail below. High-resolution graphics files must always be provided separate from the main text file.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves on a separate sheet at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

Nomenclature and Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI.

Preparation of Electronic Illustrations

- Make sure you use uniform lettering and sizing of your original artwork.
- Save text in illustrations as "graphics" or enclose the font.

- Only use the following fonts in your illustrations: Arial or Times Roman.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide all illustrations as separate files and as hardcopy printouts on separate sheets.
- Provide captions to illustrations separately.
- Produce images near to the desired size of the printed version.

Formats

Regardless of the application used, when your electronic artwork is finalised, please "save as" or convert the images to one of the following formats (Note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as "graphics".

TIFF: Colour or greyscale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (colour or greyscale): a minimum of 500 dpi is required.

DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications please supply "as is".

Please do not

- Supply embedded graphics in your wordprocessor (spreadsheet, presentation) document;
- Supply files that are optimised for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

If, together with your accepted article, you submit usable colour figures then it will be ensured that at no additional charge these figures will appear in colour on the Web (e.g., ScienceDirect and other sites) in addition to colour reproduction in print.

Captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Line drawings

The lettering and symbols, as well as other details, should have proportionate dimensions, so as not to become illegible or unclear after possible reduction; in general, the figures should be designed for a reduction factor of two to three. The degree of reduction will be determined by the Publisher. Illustrations will not be enlarged.

Do not use any type of shading on computer-generated illustrations.

Photographs (halftones)

Remove non-essential areas of a photograph. Do not mount photographs unless they form part of a composite figure. Where necessary, insert a scale bar in the illustration (not below it), as opposed to giving a magnification factor in the caption. Note that photocopies of photographs are not acceptable.

Preparation of supplementary data

Electronic supplementary material to support and enhance your scientific research is accepted as supplementary file. Supplementary files offer the Author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article. In order to ensure that your submitted material is directly usable, please ensure that data is provided in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file.

Proofs

when your manuscript is received by the Publisher it is considered to be in its final form. Proofs are not to be regarded as "drafts". One set of page proofs in PDF format will be sent by e-mail to the corresponding author, to be checked for typesetting/editing. No changes in, or additions to, the accepted (and subsequently edited) manuscript will be allowed at this stage. Proofreading is solely your responsibility.

The corrected article will be published as quickly and accurately as possible. In order to do this we need your help. When you receive the (PDF) proof of your article for correction, it is important to ensure that all of your corrections are sent back to us in one communication. Subsequent corrections will not be possible, so please ensure your first sending is complete. Note that this does not mean you have any less time to make your corrections just that only one set of corrections will be accepted.

About Sunandan Divatia School of Science

Sunandan Divatia School of Science was started in 2007 with a view to provide undergraduate and post graduate students an opportunity to be a part of the unique learning methodology of the university, which lays emphasis on academic excellence combined with industry oriented training. With the boom in information technology and more and more sophistication in instrumentation techniques, there is now a very thin dividing line between the various disciplines of science. Therefore, there is a greater need for flexibility in scientific thought as well as training manpower on an interdisciplinary plane. With this thought in view, the SVKM's NMIMS introduced, highly innovative and unique interdisciplinary courses at the School of Science from the academic year 2007-2008. The goal of the School of Science is to be a Center of Excellence in the domain of Pure and Applied Science by providing quality education and research.

Courses Offered

Ph.D. in Biological Sciences and Chemistry (Regular and Professional)

Integrated M.Sc.-Ph.D. in Biological Sciences and Chemistry

M.Sc. in Biological Sciences, Chemistry (Analytical and Organic) and Statistics

Master of Physiotherapy [In collaboration with **Nanavati Super Speciality Hospital**, Mumbai, India]

5-Year Integrated M.Sc. in Biomedical Science

B.Sc. in Applied Statistics and Analytics

Post-Graduate Diploma in Physician Assistance (2 years), **Operation Theatre Technology** (1 year), **Non-Invasive Cardiology** (1 year) and **Central Sterile Services** (1 year) [In collaboration with **Asian Heart Institute and Research Centre**, Mumbai, India]

Diploma in Clinical Research (Part time: 1 year) [In collaboration with **C. B. Patel Research Centre**, Mumbai, India]

Advanced Course in Clinical Data Management (Part time: 3 months) [In collaboration with **C. B. Patel Research Centre**, Mumbai, India]

Salient Features

Research constitutes a major thrust in all the courses offered at the School

Courses oriented to fulfill needs/demands of Research Institutions/Industry

Thrust Areas in Research

Cell Biology, Stem Cell Biology, Molecular Oncology, Reproductive Biology, Microbiology, Immunology, Pharmacology, Phytochemistry, Nanosciences, Applied Chemistry, Colloidal Chemistry and Applied Statistics

For More Information Please Contact:

School of Science, NMIMS (Deemed-to-be) University

Tel: 91-22-4235 5958/59; Fax: 91-22-2611 4512; E-mail: admissions.sos@nmims.edu;

Visit us at: http://science.nmims.edu

SVKM's
Narsee Monjee Institute of Management Studies

Deemed to be UNIVERSITY

V. L. Mehta Road, Vile Parle (W), Mumbai-400 056, INDIA.
Tel: 91-22-4235555 | **Fax:** 91-22-26114512
Email: nmims@nmims.edu | **Website:**www.nmims.edu