Chemical structure of some clinically relevant chemoprotective agents
Biomedical Research Journal

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Editorial

Dhananjaya Saranath and Aparna Khanna

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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 Malaysia 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 crossroad 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, dextrazoxane, 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 Docosahexanoic 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 linSca-1+ c-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.
Clusterin in Cancer: Dual role as a Tumor Suppressor Gene and an Oncogene

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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...
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 Alzheimer's 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 associated 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).

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.

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).

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 autoimmun 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-κB 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 downregulation 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-κB 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-κB 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-κB translocation to the nucleus, thus implying CLU to be a negative modulator of NF-κB 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-κB 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-κB pathway**

Zoubeidi et al., 2010 showed that, CLU facilitated degradation of inhibitors of NF-κB 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
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

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**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.
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 in turn 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 neurodegenerative 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 oligonucleotide, 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.
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Chemoprotectants in Cancer Chemotherapy: An Update

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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...
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

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<td>Severely toxic</td>
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<td>Myelotoxicity</td>
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<td>Peripheral nephropathy</td>
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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 (Ethylol®)

Amifostine (WR-2721, S-2-[3-aminopropylamino] ethylphosphorothioic 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
Chemoprotectants in Cancer Chemotherapy

Figure 1: Chemical structure of some clinically used chemoprotectants.

Amifostine
Dexrazoxane
Mesna
Aprepitant
Fosaprepitant

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 cells 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 G2 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 hematotoxicity (Links and Lewis, 1999). The recommended dose for amifostine is 740–910 mg/m². 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), 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 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 (Zincard®)**

Dexrazoxane (ICRF-187), a bisdioxopiperazine (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 cardiomyopathy 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
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 phosphoramid 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 phosphoramidemustard 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-hematopoietic 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., 2016).
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 T cell type 1 proinflammatory cytokines and increases helper T cell type 2 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>
<thead>
<tr>
<th>Agents</th>
<th>Therapeutic indication</th>
<th>Mechanism of action</th>
<th>Adverse reactions/Complications</th>
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<tbody>
<tr>
<td>Recombinant human erythropoietin (rhEPO)</td>
<td>Chemotherapy-induced anemia</td>
<td>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.</td>
<td>Myalgia, iron deficiency, hypertension, seizures and thromboembolism.</td>
<td>Baldo et al., 2014</td>
</tr>
<tr>
<td>GSH (γ-glutamyl-cysteine-glycine)</td>
<td>Cisplatin-induced neuropathy, renal and systemic toxicity</td>
<td>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.</td>
<td>Elevated level of glutathione in cancer cells confers resistance to chemotherapeutic agents.</td>
<td>Jena et al., 2010; Traverso et al., 2013</td>
</tr>
<tr>
<td>Sodium thiosulfate (STS)</td>
<td>Cisplatin-induced nephrotoxicity, Chemotherapy-induced extravasation injuries</td>
<td>Neutralizes chemotherapeutic agents by converting them into nontoxic species. Does not interact with intracellular concentration of chemotherapeutic agents.</td>
<td>Arthralgia, blurred vision, hyperreflexia, muscle cramps, nausea and vomiting, psychotic behaviour, tinnitus.</td>
<td>Kreidieh et al., 2016</td>
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<td>ORG-2766 (Analog of corticotropin)</td>
<td>Neuropathy induced by Paclitaxel, Vincristine, Cisplatin</td>
<td>Hypothesized that ORG-2766 mimics an endogenous peptide, which stimulates the recovery of damaged neurons.</td>
<td>No significant adverse effects reported</td>
<td>Hershman et al., 2014</td>
</tr>
<tr>
<td>5-Methylselenocysteine (MSC)</td>
<td>Cisplatin-induced hematological, renal and ototoxicity</td>
<td>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.</td>
<td>Mild toxicity in liver and kidneys.</td>
<td>Bhattacharya, 2011</td>
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Contd...
### Table 2: Clinically relevant chemoprotective agents (Contd...)

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<tr>
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<tbody>
<tr>
<td><strong>α-Tocopherol (Vitamin E)</strong></td>
<td>Chemotherapy-induced systemic toxicity, especially peripheral neuropathy</td>
<td>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.</td>
<td>Nausea, diarrhea, stomach cramps, blurred vision, rash, bruising and bleeding. α-tocopherol may increase the possibility of hemorrhagic stroke in brain.</td>
<td>Nakayama et al., 2011</td>
</tr>
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### Table 2: Clinically relevant chemoprotective agents (Contd…)

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<tbody>
<tr>
<td>Vitamin C (Ascorbic acid)</td>
<td>Chemotherapy-related symptoms, such as fatigue, insomnia, loss of appetite, nausea, and pain.</td>
<td>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, IL-2, IL-6, IL-8, in cancer patients.</td>
<td>Indigestion, diarrhea and skin rashes. Vitamin C interferes with antitumor activity of methotrexate, dacarbazine and doxorubicin.</td>
<td>Carr et al., 2014</td>
</tr>
<tr>
<td>Melatonin (N-acetyl-5-methoxy tryptamine)</td>
<td>Chemotherapy-induced systemic toxicity</td>
<td>Melatonin eliminates free radicals, and also induces production of antioxidant enzymes. Melatonin is immunomodulatory and endocrine-modulatory.</td>
<td>Headaches, dizziness, nausea and drowsiness</td>
<td>Seely et al., 2012</td>
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Table 3: Promising preclinical chemoprotective compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
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<tbody>
<tr>
<td>Epigallocatechin-3-gallate (ECGC)</td>
<td>Cyclophosphamide-induced systemic toxicity and DNA damage</td>
<td>Acts as an antioxidant, reduces lipid peroxidation and genotoxicity</td>
<td>Sai Sampath et al., 2011</td>
</tr>
<tr>
<td>Selenium nanoparticle (Nano-Se)</td>
<td>Cyclophosphamide-induced hepatotoxicity, pulmonary toxicity and genetic damage</td>
<td>Mitigates oxidative stress, DNA damage and enhances antioxidant status</td>
<td>Bhattacharjee et al., 2014; Bhattacharjee et al., 2015</td>
</tr>
<tr>
<td>Indole-3-carbinol (I3C)</td>
<td>Cyclophosphamide-induced developmental toxicity and teratogenicity</td>
<td>Attenuates limb malformation and tail malformation</td>
<td>Bailey et al., 2005</td>
</tr>
<tr>
<td>Resveratol</td>
<td>Doxorubicin-induced cardiotoxicity</td>
<td>Ameliorates activity of Na⁺, K⁺-ATPase and antioxidant enzymes</td>
<td>Tatliyedde et al., 2009</td>
</tr>
<tr>
<td>Crocin</td>
<td>Doxorubicin-induced myocardial toxicity</td>
<td>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.</td>
<td>Elsherbiny et al., 2016</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>Doxorubicin-induced testicular toxicity</td>
<td>Prevents oxidative stress, DNA damage and apoptosis by reducing expression of NF-κB, p38 and caspase-3.</td>
<td>Trivedi et al., 2011</td>
</tr>
<tr>
<td>Edarabone</td>
<td>Doxorubicin-induced cardiomyopathy</td>
<td>Improves conduction abnormalities, arrhythmia and myocardial ischemia</td>
<td>Xin et al., 2011</td>
</tr>
<tr>
<td>Diphenylmethyl selenocyanate (DMSE)</td>
<td>Cisplatin-induce nephrotoxicity</td>
<td>Enhances activity of antioxidant enzymes and inhibits expression of proinflammatory COX-2 and iNOS.</td>
<td>Chakraborty et al., 2011</td>
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Contd...
Table 3: Promising preclinical chemoprotective compounds (Contd…)

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

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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.

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Email: lslimaye@nccs.res.in

Review

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

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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-1^-c-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
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. \( \omega-3 \) (n-3) and \( \omega-6 \) (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
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 ⁶⁰Co 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, 10⁶ 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, 10⁶ 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.
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
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 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.
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
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.

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Mathematical Modeling of Viral Epidemics: A Review

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Mathematical models to describe transmission and propagation of diseases have gained momentum over the last hundred years. Formulated mathematical models are currently applied to understand the 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 (Abidoret 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.
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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 involves 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 output compared 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 Bernoulli (Brauer, 2009; Bernoulli, 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 \( \beta \). 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 \( \alpha \), then the mean transmission period in any individual is given by \( 1/\alpha \). 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

\[
\begin{align*}
\frac{dS(t)}{dt} &= -\beta S(t) I(t) \\
\frac{dI(t)}{dt} &= \beta S(t) I(t) - \alpha I(t) \\
\frac{dR(t)}{dt} &= \alpha I(t)
\end{align*}
\]  
(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...
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 $\frac{\beta N}{\alpha}$ 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 from the cumulative incidences data in the initial growth phase of the outbreak, as:

$$R_0 = \left(1 + \frac{r}{\alpha}\right)$$  \hspace{1cm} \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) $\mu$, the Eqn1.1 can be modified as

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

$$\frac{dI(t)}{dt} = \beta S(t)I(t) - \alpha I(t) - \mu I(t)$$  \hspace{1cm} \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.

approximation of seasonally forced transmission rate is
\[ \beta(t) = \beta_0(1 + A \cos 2\pi t) \] (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 \( \kappa \) 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 to susceptible members of the population with a mean transmission rate \( \beta \). 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 \( \alpha \), then the mean transmission period in any individual is given by \( 1/\alpha \). Fig. 2 describes schematically the SEIR model of disease transmission. Considering the constant population size \( N = S + E + I + R \), the set of
differential equations describing the transmission as per the basic SEIR model is given by

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

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

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

\[
\frac{dR(t)}{dt} = \alpha I(t)
\]  \hspace{1cm} (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:

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

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

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

\[
\frac{dN(t)}{dt} = -(1-f) \alpha I(t)
\]  \hspace{1cm} (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 \( (t_o \text{ and } y_o) \), respectively, using the equation (Fraser \textit{et al.}, 2009),

\[
y = y_o e^{r(t-t_o)}
\]  \hspace{1cm} (Eqn. 2.3)

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

\[
R_o = \left( 1 + \frac{r}{\alpha} \right) \left( 1 + \frac{r}{\kappa} \right)
\]  \hspace{1cm} (Eqn. 2.4)

with the mean infective period \( 1/\alpha \) and mean incubation period \( 1/\kappa \). This gives a more accurate estimation of the \( R_o \), compared to the SIR model, where the latent phase was not considered. This is best explained with the help of an example. Gurav \textit{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
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) \( (I) \) compartment at 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 \( \beta N \) others per unit time, where \( \beta \) is the transmission rate. vii) A fraction \( \alpha \) of the infective individuals and a fraction \( \eta \) 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):

\[
\begin{align*}
\frac{dS}{dt} &= -\beta S(I(t) + \eta A(t)) + \alpha I(t) + \alpha A(t) \\
\frac{dE}{dt} &= \beta S(I(t) + \eta A(t)) - \beta E(I(t) + \eta A(t)) - kE \\
\frac{dI}{dt} &= kE - \beta (I(t) + \eta A(t)) - \alpha I(t) \\
\frac{dA}{dt} &= kE - \beta (I(t) + \eta A(t)) - \alpha A(t) \\
\frac{dR}{dt} &= \alpha I(t) + \alpha A(t)
\end{align*}
\]
The doubling time (the time period in which the size of the outbreak doubles) as calculated from 

\[ t_d = \frac{\ln(2)}{r} \]

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 being 0.001566. The doubling time (the time period in which the size of the outbreak doubles) as calculated from 

\[ t_d = \frac{\ln(2)}{r} \]

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 \( R_e = 2.61 \) and transmission rate (\( \beta \))

\[
\begin{align*}
\frac{dS}{dt} &= -\beta S(I + qA) \\
\frac{dE}{dt} &= \beta S(I + qA) - kE \\
\frac{dI}{dt} &= pkE - \alpha I \\
\frac{dA}{dt} &= (1 - p)kE - \eta A \\
\frac{dR}{dt} &= \alpha I + \eta A \\
\frac{dC}{dt} &= \alpha I
\end{align*}
\]

(Eqn. 3.1)
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 $\beta$ 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 $\rho$ of the latent individuals ($0 < \rho < 1$) develop symptoms and become Infective at the rate $\kappa$ and the rest ($1-\rho$) progress to become asymptomatic $A(t)$ also at the same rate $\kappa$. Asymptomatics proceed to recovered $R(t)$ class at the rate $\gamma_1$. The infectious individuals are diagnosed and hospitalized at rate $\alpha$, while some recover with hospitalization at rate $\gamma_2$ and die at the rate $\delta$. The transmission is described by the following set of differential equations:

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

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

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

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

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

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

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

$$\frac{dC}{dt} = \alpha I(t)$$

(Eqn. 4.1)

Here, $\mu$ 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 \( \beta, \gamma, q, \alpha, \) 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 (Coburnet al., 2009; Galvanicet 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 MacDonald. 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 \( a \) 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 \( a/H \). 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 \(\tau\), then it is possible that some individuals might recover or do not get the disease during this incubation period. Thus, of those individuals infected \(\tau\) unit times ago, only a proportion 
\[
\left(\frac{\alpha}{H}\right)b[H - X(t - \tau)]Y(t - \tau)\exp(-r\tau)
\]
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

\[
\frac{dx}{dt} = -rx(t) + \left(\frac{\alpha}{H}\right)b[H - X(t - \tau)]Y(t - \tau)\exp(-r\tau)
\]
(Eqn. 5.1)

Let \(\mu\) 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 \(\tau\), 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:

\[
\frac{dy}{dt} = -\mu y(t) + \left(\frac{\alpha}{H}\right)cX(t - \tau)(M - Y(t - \tau))\exp(-\mu\tau)
\]
(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

\[
x(t) = \frac{X(t)}{H}, \quad y(t) = \frac{Y(t)}{M}
\]

and

\[
m = \frac{M}{H}.
\]

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

\[
\frac{dx}{dt} = rx(t) + \alpha b m(1 - x(t - \tau))y(t - \tau)\exp(-r\tau)
\]
\[
\frac{dy}{dt} = \mu y(t) + \alpha c m x(t - \tau)(1 - y(t - \tau))\exp(-\mu\tau)
\]
(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:

\[
R_0 = \frac{\alpha^2 bcm}{r\mu} e^{-\tau\mu} e^{-\tau\mu}
\]

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 \( \tau_i \), the average number of mosquito bites received from \( m \) susceptible mosquitoes, each with a biting rate \( \alpha \), gives a total of \( \frac{\alpha c_m e^{-r \tau_i}}{r} \) mosquitoes infected by the primary human case. Each of these mosquitoes survives for an average time \( 1/\mu \) and with another incubation period \( \tau_j \) in mosquitoes, makes a total of \( \frac{\alpha c_m e^{-\mu \tau_j}}{\mu} \) infectious bites. The total number of secondary cases is thus estimated to be \( \frac{\alpha^2 b c m e^{-r \tau_i} e^{-\mu \tau_j}}{r \mu} \) which is (2). The parameter \( \alpha \) 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 Montserrat 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 Montserrat. The estimated value of basic reproductive number for EVD in Montserrat 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.

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Recent Advances in the Treatment of Malaria

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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...
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. falciparum 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.

Figure 1: Different stages in the malarial parasite life cycle (NIAID, 2015).
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 + pyrimethamine) 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
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
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 antimalarials - 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 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 chemoprotection. DDD498 was developed from a screening programme against blood-stage malaria parasites. This drug targets

![Figure 3: Compounds currently in preclinical stages.](image-url)
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<sub>50</sub> < 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<sub>50</sub> 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<sup>+</sup> 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 $\text{Na}^{+}$ 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 dihydriisoquinolones (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 $\text{Na}^{+}$ 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 cryptopsis (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
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.
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Biomagnetic Interaction of Functionalized Iron Oxide Nanoparticles with Bovine Serum Albumin

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Functionalized iron oxide (magnetic) nanoparticles are promising candidates for detection and sensing of target molecules 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), polyethylene 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

Keywords: Magnetic nanoparticles, bio-magnetic sensors, AC susceptibility, macromolecules, Brownian relaxation.

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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$_2$O), ferrous chloride tetrahydrate (FeCl$_2$.4H$_2$O), 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$_2$O and 1.05 g of FeCl$_2$.4H$_2$O 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$^{-}$ + Fe$_3$O$_4$ + H$_2$O 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\(^{-1}\), 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\(_3\)O\(_4\) nanoparticles takes place through the amine functionality. Possibly, amine groups of GC form complexes with the Fe-atoms on surface of Fe\(_3\)O\(_4\) 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\(_3\)O\(_4\) 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\(_3\)O\(_4\) nanoparticles by sulfonate functionality.

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\(_3\)O\(_4\) (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° 20 respectively. The data indicates formation of single-phase Fe\(_3\)O\(_4\)
Figure 1: Optimal conditions for efficient transduction of NSC

(A) Concentration and length of exposure for maximum viability and transduction for rat NSC (i-iii) and human NSC (iv-vi) was determined following incubation of cells with GFP BacMam for 60 minutes (dotted line) or overnight (dashed line). NSCs exposed to different concentrations (v/v) of GFP BacMam were harvested after 24 hours and cells were analyzed by flow cytometry to determine viability of cells based on forward and side scatter, percent transduction based on %GFP positive cells and intensity of GFP expression in the transduced cells. Rat NSC showed no significant toxicity with BacMam virus treatment between 1-20% v/v virus either at 60 minutes or with overnight incubation of cells with the virus (i). Greater than 50% of the cells were transduced both with 60 minutes and overnight incubation of cells with 1% v/v virus with increase in percentage of GFP positive cells up to 80% with increasing virus concentrations (ii). The intensity of GFP in the transduced cells did not significantly increase from 5% virus to 20% treated cells for both 60 minutes and overnight incubation conditions (iii). In contrast human NSC incubated with varying concentration of BacMam showed more sensitivity to the presence of virus with 60 minutes of incubation relatively better for cell viability than overnight incubation (iv). The surviving cells however showed a linear increase in %GFP positive cells with increasing concentrations of the virus with overnight incubation resulting in higher percentages of GFP positive cells than cells transduced overnight (v). The intensity of GFP also showed a corresponding higher GFP intensities in cells treated with higher percentage virus overnight (vi).

(B) Optimal conditions determined for rat NSC transduction (i) was 60 minutes incubation with 20% virus, and overnight incubation with 5-10% v/v virus for human NSC (ii) with the virus added directly added to adherent cells cultured in StemPro NSC media.

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

The thermogravimetric analysis (TGA) of bare Fe$_3$O$_4$, GC-MNPs, PEGME-MNPs and the PSSNa-MNPs are shown in Fig. 3, indicating one weight loss process in Fe$_3$O$_4$. 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 250°C. 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 [Karaöglu 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 structures
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$_3$O$_4$.

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 of PEGME-MNPs. The crystallite in the image has d-spacing of 2.5 Å corresponding to the (331) plane of Fe$_3$O$_4$. In PSSNa-MNPs, HRTEM image shows the crystallite d-spacing is 2.93 Å corresponding to the (220) plane of Fe$_3$O$_4$.

**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 ($\omega$) was recorded. The magnetic response exhibited was expressed by a complex magnetic susceptibility $\chi$.

The imaginary part of the complex magnetic susceptibility ($\chi''$) corresponds to the out-of-phase response and is expressed as

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

Where $\chi_0$ is the DC magnetic susceptibility and $\tau$ is the effective magnetic relaxation time of MNPs.

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

**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.

<table>
<thead>
<tr>
<th>Functionalized MNPs</th>
<th>Initial Diameter of MNPs (TEM)</th>
<th>Diameter after immobilizing BSA from AC susceptibility results</th>
<th>Increase in size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC – MNPs</td>
<td>11.41 nm</td>
<td>45.29 nm</td>
<td>33.87 nm</td>
</tr>
<tr>
<td>PEGME – MNPs</td>
<td>12.91 nm</td>
<td>42.51 nm</td>
<td>29.60 nm</td>
</tr>
<tr>
<td>PSSNa – MNPs</td>
<td>13.62 nm</td>
<td>42.02 nm</td>
<td>28.40 nm</td>
</tr>
</tbody>
</table>
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).

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