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Quaternary ammonium palmitoyl glycol chitosan-based nano-doxorubicin delivery system: Potential applications for cancer treatment and theranostic

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Abstract

Recently, the doxorubicin loaded Quaternary Ammonium Palmitoyl Glycol Chitosan (DOX-GCPQ) nanoformulation for DOX delivery and non-invasive monitoring of DOX accumulation and biodistribution at tumor site utilizing DOX's self-florescent property was evaluated. DOX-GCPQ amphiphilic polymeric nanoformulations were prepared and optimized using Artificial Neural Network (ANN), and like other particulate delivery systems, optimized formulation has been characterized by atomic force microscopy, dynamic light scattering, FTIR and XRD. Such systems are to be investigated for *in-vitro* release, cellular, tumor and tissue uptake, thus ANN-optimized nanoformulation has been tested for above. Since nanoformulation, on accounts of smaller size and higher surface to volume ratio alters its biological behavior and encapsulated therapeutic agent, newly developed system has also been assessed for its toxicity and safety. Optimized DOX-GCPQ has been investigated for *in-vitro* biocompatibility, apoptosis and hemo-, nephro-, hepato- and cardio- toxicities in mice. Small particle size, sustained drug release, enhanced cellular uptake, potential to target tumor and its monitoring by optical imaging has indicated DOX-GCPQ as an efficient nanotheranostic system which may also work as future safe drug carrier system with reduced DOX-induced organ toxicity.

Introduction

Cancer is a leading cause of death worldwide. Until 2030, approximately new cancer cases would be twenty six million and seventeen million cancer related deaths per year are expected [1]. Common cancer types included carcinoma, lymphoma, sarcoma and leukemia. Prognosis of cancer is still miserable despite advancements in earlier diagnosis, surgical and non-operative methodologies. Major treatment strategies for cancer therapy include surgery, chemotherapy radiation therapy, immunotherapy, photothermal therapy, photodynamic therapy, individually or any combinations of above. Chemotherapy is administration of chemotherapeutic agents (Table 1).

Table 1: Classification, with examples of chemotherapeutic agents

Alkylating Agents	Antimetabolites	Antitumor Antibiotics	Plant Alkaloids
Cisplatin	5-Flurouracil	Doxorubicin	Vinblastine
Cyclophosphamide	Methotrexate	Bleomycin	Vincristine
Busulfan	Capecitabine	Mitomycin-c	Paclitaxel
Ifosfamide	6-Mercaptopurine	Mitoxantrone	Docetaxel
Lomustine	Gemcitabine	Dactinomycin	Etoposide

Doxorubicin Hydrochloride (DOX) is one of the agents which is widely used against number of malignancies including solid tumors, transplantable leukemias and lymphomas [2]

Doxorubicin (DOX)

Chemicaly doxorubicin hydrochloride is (8S:10S)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxohexopyranosyl)-oxy]-8-glycoloyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride having molecular formula of $C_{27}H_{29}NO_{11}$.HCl and molecular mass of 579.98. Chemical structure of DOX HCl in Figure 1. It is odorless, hygroscopic powder, red-orange in color, and soluble in water, dilute alcohols.

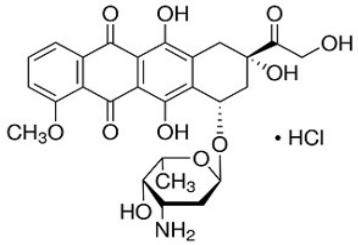

Figure 1: Chemical structure of DOX

Conventional DOX HCl is rapidly cleared from plasma after intravenous administration and demonstrates substantial tissue binding. 40-50% of given dose is excreted via biliary route while 4-5% of administered dose follow urinary path. Plasma and tissue accumulation of DOX is resulted from impaired liver function causing its slow excretion. DOX does not cross blood brain barrier. DOX, despite of its side-effects is known for its efficacy. Therapeutic uses of DOX (Table 2).

Table 2: Therapeutic uses of DOX

Type of cancer	Therapeutic Use
Breast cancer	First line therapy
Lung cancer	Squamous sarcoma, Adenosarcoma, Carcinoma
Leukemia	Acute lymphoblastic and Non-lymphoblastic leukemia
Other type of tumors	Carcinoma of soft tissues, Osteogenic sarcoma, Kaposi's sarcoma
Tumors in pediatric patients	Hepatocellular carcinoma, Testicular cancer

DOX inhibits macromolecular biosynthesis by interfering with intercalation of DNA. It also inhibits topoisomerase II, key enzyme in DNA replication. DOX. HCl also generates free radicals that cause DNA and cell membrane damage [3]. DOX has side effects which can be grouped into different categories (Table 3).

Table 3: DOX Adverse Effects

Side effect	Adverse effects
Cardiotoxicity	Acute effects: Arrhythmia, Tachycardia Chronic effects: Cardiomyopathy
Nephrotoxicity	Atrophy of Glomerulous, Increased Permeability of Glomerulous
Hematological	Bone marrow depression
Symptomatic Effects	Oral ulceration, Skin pigmentation

Issues associated with DOX

Current cancer chemotherapeutic agents exhibit inability to cross biological barriers, non-specifically distributes to normal cells, poorly biodistributed to region of interest, have poor efficacy, show drug resistance, and lack theranostic ability which combines possibility to treat and monitor the progress of therapy [4]. DOX, despite having broad spectrum anti-neoplastic activity, distributes nonspecifically, leading to side effects like hepatotoxicity, nephrotoxicity and particularly cardiotoxicity which have limited its clinical use. Furthermore, little amount of DOX reaches to region of interest (tumor), necessary for therapeutic action due to rapid plasma clearance and excessive excretion (40%) of drug and metabolites after hepatic metabolism [5].

Addressing issues associated with DOX by formulation design

Several approaches have been employed in order to overcome biological barriers, non-specific delivery, poor biodistribution and toxicity of DOX, like other chemotherapeutic agents and at their simultaneous ability for treatment monitoring [6]. The approaches to address above limitations are managing prolonged schedules of infusion, simultaneous administration of cardioprotective agent, i.e., dexrazoxane and use of anthracycline analogs and developing new derivatives of chemotherapeutics that can also monitor tumor progression. Modification of existing therapeutic moieties through drug delivery technologies is also an option which is preferred over other strategies [7]. As a result, several, usually nanocarrier-based DOX delivery systems have been attempted to avoid systemic toxicity of DOX alter distribution and ultimately reduce accumulation in normal organs [4, 8].

Nanomedicine is nanosized system comprising nanocarriers and the active moieties that can be used for diagnosis as well as treatment of diseases. Nanomedicine demonstrate enhanced bioavailability and drug delivery at site of action without excipient-associated side effects and with reduced toxicity [9]. These nanocarrier systems are liposomes or polymeric nanocarriers. Polymeric DOX nanoformulation developed using non-toxic, biocompatible and biodegradable material is a promising approach to achieve enhanced efficacy, lessen its side-effects and reduced cytotoxicity by delivering drug at specific site [10]. Literature cites several methods for preparation of polymeric nanoparticles including physical, chemical, and biological methods. Compared to traditional methods, sonication is simple, single-step, convenient, and relatively inexpensive method. Ultrasonic method is green synthetic approach, which is safe for environment and provides number of advantages in process development and manufacturing as well as product design. Sonication method adopted for development of GCPQ nanoformulation was coupled with development of nanomedicine of GCPQ-DOX without requiring addition of any excipients or other substances [11].

Quaternary ammonium palmitoyl glycol chitosan

Amphiphilic Quaternary Ammonium Palmitoyl Glycol Chitosan (GCPQ) glycol chitosan derivative (Figure 2). These polymers self-assemble in aqueous media are known as polysoap and drug solubilizing agents [12]. GCPQ nanoparticles have shown great potential as delivery system, which increases transport of hydrophobic and hydrophilic drugs across GIT epithelium, cornea and brain-blood barrier [13] . GCPQ self-assembles into stable nano (colloidal) size particles without use of chemical cross

linker or ionic gelation agents. Critical micellar concentration CMC of GCPQ is low, thus nanoparticles does not prematurely disaggregate in body fluid and GCPQ nanoparticles possess permeation enhancer properties thus, reach target site [14]. GCPQ is biodegradable, biocompatible and non-toxic polymeric nanocarrier which avoids uptake by liver and spleen and target tumor passively [13]. Drug encapsulation inside GCPQ polymer protect their degradation from gastric and intestinal environment leading to improved gut absorption of active therapeutic agents. GCPQ nanoparticles also promote drug dissolution and also reported for delivery of drugs from oral or intravenous route of administration into tumors [15].

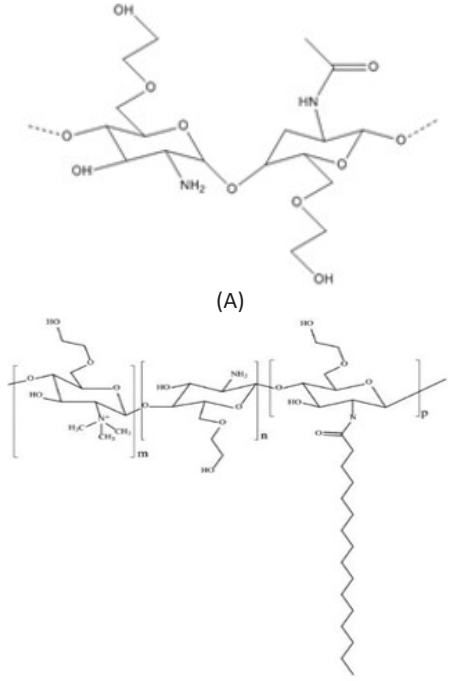

Figure 2: (A) Glycol chitosan (B) Quaternary Ammonium Palmitoyl Glycol Chitosan

Effective cancer treatment, diagnosis and prognosis potentials of GCPQ-DOX

For effective cancer diagnosis and prognosis, non-invasive methods are required yet current diagnostic and therapeutic modalities are invasive, non-specific and are associated with other issues. Thus, to address above critical challenges recently development of theranostic has drawn tremendous attention. To develop an effective theranostic of an existing chemotherapeutic agent having already established treatment efficacy requires overcoming issues associated with agent and inducing a ligand/exploring features which can be employed for monitoring of progress of therapy. DOX is considered to fulfil both above characteristics, an established chemotherapeutic moiety and also being self-florescent [16]. Nevertheless, DOX also demonstrates systemic toxicity in different organs [17]. Even FDA approved pegylated and non-pegylated liposomal formulations (Doxil and Myocet, respectively), though target tumor more effectively with reduced cardiotoxicity, alopecia, and neutropenia but has raised toxicity referred to as hand-foot syndrome [18]. Thus, there is still a need to develop new and effective theranostic system. Previously, no data exist about systemic delivery of DOX loaded GCPQ polymeric nanoformulation. It was hypothesized that DOX-GCPQ can exhibit enhanced bioavailability, desired biodistribution, enhanced efficacy, without toxicity and better drug accumulation at target site along with exploring imaging potential of DOX. Following schematics in Figure 3 has been employed for development and characterization of DOX-GCPQ.

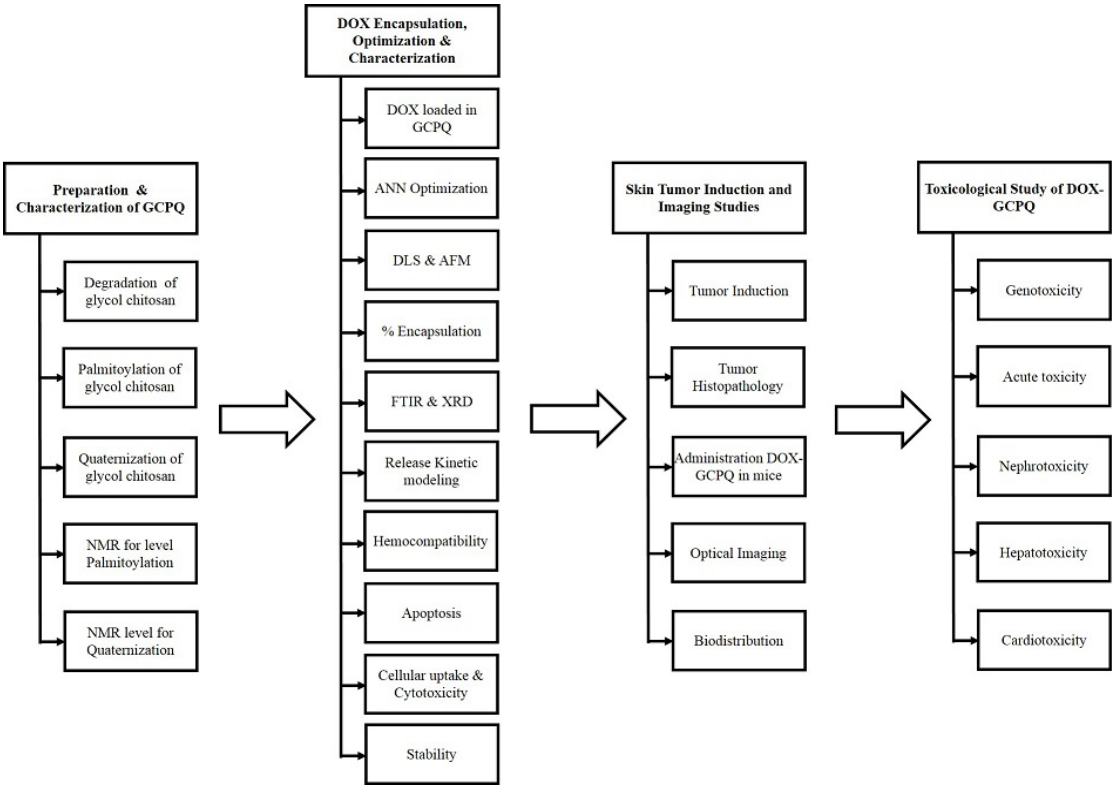

Figure 3: Schematics for development and characterization of new DOX nanoformulation

DOX-GCPQ nanoformulation

DOX has successfully been encapsulated in GCPQ using probe sonication and optimized using Artificial Neural Network (ANN) for smaller particle size, minimum PDI, higher surface charge and minimized variation (SD) for size and PDI. ANN-optimized DOX-GCPQ is anionic spherical micelles with hydrodynamic particle size of less than 100 nm and demonstrates sustained release pattern over 48 h. Weibull and Korsmeyer-Peppas models support DOX release from a matrix type carrier and was diffusion/erosion, respectively. As compared to DOX, higher DOX-GCPQ uptake has been noted, using Fluorescence microscopy in human Rhabdomyosarcoma (RD) cells. In *in-vitro* cytotoxicity assay significant cytotoxicity of DOX-GCPQ against RD cells with lower IC_{50} is indicated as compared to DOX and blank GCPQ.

In-vivo tumor imaging can be used for an early cancer detection and monitoring of cancer therapy. In skin tumor xenografts, optical imaging reveals significantly lower DOX-GCPQ in heart and liver but exhibited accumulation mainly in tumor as compared to other tissues. Earlier, clinical applications of DOX are limited by systemic toxicity, including cardiotoxicity, hepatotoxicity and nephrotoxicity mediated by oxidative stress, lipid peroxidation [19]. Thus, any system developed for DOX, including DOX-GCPQ nanoformulation, must be investigated for toxicity profile before their biological application [20]. DOX-GCPQ demonstrates high *in-vitro* hemocompatibility with significant apoptotic potential and negligible genotoxicity, lesser than DOX. DOX-GCPQ exhibits significantly lesser toxicity in terms of body weight, organ weight, body weight mass index and haematology. Drug induced nephrotoxicity, hepatotoxicity and cardiotoxicity, as indicated via elevation of serum biomarkers, reduced antioxidant enzymes level and distorted histopathology of kidney, liver and degenerative changes in heart tissue have not been observed in DOX-GCPQ nanoformulation.

Envisioned applications of the developed system

DOX-GCPQ- a better therapeutic option

GCPQ nanocarrier can successfully be synthesized, characterized and encapsulated DOX into nanocarrier. Optimized nanocarrier releases DOX in sustained pattern, biocompatible with human RBCs with significant *in-vitro* apoptotic potential at lower IC_{50} along enhanced cellular uptake against RD cells. The system also demonstrates better drug localization at tumor site in skin tumor mice model, lesser organ toxicity and genotoxicity. Thus, DOX-GCPQ may be a promising biocompatible nanoformulation for clinical applications.

Potential use of DOX-GCPQ as nanotheranostic

Since DOX has florescent property, when encapsulated in GCPQ, it also shows an appropriate biodistribution in tumor thus, system can also be used for tumor imaging as nanotheranostic. Nanosystems with such properties as DOX-GCPQ has shown may exhibit the potential to improve loco regional disease control and cure rates across a diverse range of tumor types. Thus, therapeutic effect could be judged by monitoring progression of disease or tumor volume at different time points simultaneously with enhanced therapeutic effect of anticancer drug at minimum dose for optimal effect. Combining novel molecularly-targeted imaging agents and drugs for detection and therapeutic application will open new arena in the nanotheranostics, an emerging research field.

Ultrasound mediated drug delivery

DOX-GCPQ nanoformulation can be explored to deliver DOX via ultrasound mediated polymeric micelle drug delivery. Systemic toxicity and low delivery efficiency of conventional chemotherapy require improved targeted drug delivery at site of action. Drug delivery via ultrasound is a non-invasive stimuli responsive modality to deliver therapeutic agent at site of action and polymeric micelles are potential candidate for this modality. Ultrasound mediated drug delivery system has potential to reduce toxicity with better therapeutic response.

Future prospects

DOX-GCPQ has opened new horizons for cancer therapy with earlier diagnosis real time non-invasive drug delivery and monitoring simultaneously. Certain aspects given below, have not been studied for the GCPQ-DOX.

Pharmacokinetic study of DOX-GCPQ

Pharmacokinetic studies primarily are aimed to quantify parameters of drug absorption, distribution, metabolism and excretion from blood level time curve, accomplished through measuring concentration of drug in blood at several time intervals. Blood level time curve could be related to Minimum Effective Concentration (MEC), which provides information on duration and intensity and efficacy of drug. A relation with Minimum Toxic Concentration (MTC), blood level time curve indicates accumulation and disposition, and thereby safety of drug [21]. Furthermore, a longer half-life is generally observed with colloidal carriers which may leads to drug accumulation. So, Total Clearance (CL_T) and Volume of Distribution (Vd) could be assessed through pharmacokinetics studies. Pharmacokinetic studies are basis for calculation of dose (loading as well as maintenance), dosage regimen and appropriate and rational drug use, hence are important in drug developmental process and beyond [22]. Pharmacokinetic studies of DOX-GCPQ nanoformulation in appropriate animal models or humans may help to compare bioavailability and pharmacokinetics of newly developed DOX nanoformulation to that of free drug as well as existing FDA approved commercial DOX preparations.

Single cell pharmacokinetic studies of DOX-GCPQ

Pharmacokinetic studies at organ level provide information about drug biodistribution in body though cannot explain drug action at cellular level. Current pharmacokinetic models cannot provide insight about heterogeneity of single cell behavior, may be an issue for anticancer drugs, as tumor cells exhibit cell-to-cell variability. Within cancer cells drug therapeutic response can varied owing to resistance mechanism, tumor microenvironment influences and fluctuations in target molecules. Therefore, due to existence of such heterogeneity in tumor tissues models, single-cell dynamics might be more predictive [23]. Single cell *in-vivo* imaging enables to analyze tumor microenvironment, measurement of tumor heterogeneity, drug dose optimization, determination of concentration of drug for therapeutic outcome, extrapolation of models to humans and detailed analysis of failure. So, single cell pharmacokinetic studies evaluate cell signaling path analysis and accumulation of drug for therapeutic outcome can also be planned for DOX-GCPQ using intravital microscopy to track drug biodistribution in individual cells.

Tissue biodistribution studies of DOX-GCPQ

Pharmacokinetic and tissue biodistribution monitoring of nanoparticles are significant features to consider during de-

velopment of nanoparticles. Tissue distribution of nanoparticles is dependent on size, shape, surface functionalization of nanoparticles, upon *in-vivo* application of nanoparticles tissue distribution influenced toxicity of nanoformulations. Nanocarrier based drug delivery systems presents controlled biodistribution having ability to avoid toxic effects [24]. Tissue distribution studies may help to tissue drug disposition profiles, characterize pattern of concentration built in in tissues and disposition and/or degree of transient drug retention/tissue binding. Tissue distribution studies show nature of correlation between organ/tissue drug concentration with plasma concentration, appreciating uptake mechanism [25]. Such study would provide information regarding tissue drug accumulation and also support and confirm *in-vitro* and *in-vivo* DOX localization in tissue using florescent imaging.

Conformation of theranostic applications of DOX-GCPQ

DOX has been reported to possess a self-florescent property and has showed an appropriate biodistribution in tumor via optical imaging, thus theranostic potential of DOX-GCPQ has been noted. In future therapeutic effect monitoring studies should be carried out in an appropriate animal tumor model at different time points and the progression of disease with regression of tumor volume should be noted with enhanced therapeutic effect of DOX-GCPQ nanoformulation at minimum dose for optimal effect. Theranostic systems can be tailored for personalized medicines and are considered one among important treatment modality for cancer [26].

Investigation of renal accumulation of DOX after administration of DOX-GCPQ

DOX do not significantly localize in heart, liver and spleen after the administration of GCPQ-DOX nanoformulation, in line with previous study [27]. However, significant DOX accumulation in kidney was noted after administration of DOX-GCPQ, which might be ascribed to the excretion of DOX through kidney, rather than through biliary route, reported for excretion of free DOX [28]. Histopathological examination of kidney tissues exhibited a mild vascular congestion but at same time supported preservation of normal renal architecture after administration of DOX-GCPQ due to GCPQ's antioxidant potential. To conclude whether DOX accumulation after administration of DOX-GCPQ was due to an altered route of excretion through kidney, accumulation or altered biodistribution, detailed investigations are warranted.

Chronic toxicity studies of DOX-GCPQ

DOX-GCPQ nanoformulation acute toxicity studies in Balb/c mice have been conducted to investigate cardio-, nephro- and hepato-toxicity for period of 14 days. It warrants that detailed chronic toxicity study in appropriate animal may be conducted with long term exposure of DOX-GCPQ nanoformulation.

Deep tumor biodistribution studies of DOX-GCPQ

DOX-GCPQ shows promising DOX biodistribution and localization in skin tumor induced in mice model. DOX has antitumor activity against wide range of tumor including deep tumors. Therefore, in future a study on distribution of drug in xenograft model for breast as well as ovarian cancer cells can be carried out to investigate therapeutic potential of DOX-GCPQ in deep tumors.

Clinical investigation of DOX-GCPQ nanoformulation

After pharmacokinetic, theranostic and toxicity studies in appropriate animals, DOX-GCPQ nanoformulation could be clinically investigated for appropriate bioavailability tissue distribution along with the reduced toxicity profile in patients.

Accessing expanded therapeutic potentials of DOX-GCPQ

The conventional DOX has been indicated in solid tumors, hepatocellular carcinoma, and testicular cancer of pediatrics, transplantable leukemias and lymphomas, pegylated DOX liposomal formulations have been employed in ovarian, and breast cancers, while the non-pegylated thermo-sensitive DOX is used for hepatocellular carcinoma. Thus, DOX in GCPQ nanocarrier should be studied in detail for any altered biodistribution in organs as compared to conventional as well as liposomal preparation for its expanded therapeutic potentials for cancers apart from approved currently.

Use of GCPQ as nanocarrier for other chemotherapeutic agents

GCPQ could be employed for addressing the issues associated with other chemotherapeutic drugs such as sorafenib, 5-Fluorouracil (5-FU), imatinib etc. Sorafenib, a multi-tyrosine kinase inhibitor has low bioavailability requiring large dose leading to gastrointestinal problems, hypertension and systemic toxicity. Amphiphilic polymeric micelles could be employed to overcome limitations associated with sorafenib treatment. GCPQ is able to spontaneously self-assemble to give micellar nano sized clusters and having higher drug incorporation in micellar clusters leading to enhanced bioavailability of hydrophobic drugs [12]

5-Fluorouracil member of antimetabolites is widely used in treatment of digestive system neoplasms, however its clinical use is accompanied by several side effects including poor drug absorption, short half-life (10-20min), rapid drug clearance and non-selective drug action on healthy cells [29]. GCPQ increases drug transport of both hydrophilic and hydrophobic drug moieties across epithelium of gastrointestinal tract, cornea and blood brain barrier providing rapid drug dissolution and promoting drug transport across gastrointestinal epithelium leading to prolonged contact of nanoparticles with GIT enterocytes [27]. Thus, the system could also be tried for 5-FU and other anticancer agents.

Imatinib show poor brain distribution, this cannot be used for treating brain gliomas due to efflux transporters in Blood Brain Barrier (BBB) for which imatinib is substrate leading to imatinib resistance [30] . GCPQ have potential to prevent plasma degradation of peptide after intravenous administration and allow adherence to endothelial brain cells, aiding peptide delivery across BBB [15]. Since GCPQ is able to cross BBB, thus could be employed as a nanocarrier for imatinib.

Use of GCPQ as nanocarrier for drugs non-chemotherapeutic drugs

There are several features of chitosan derivative, GCPQ which could favor its use for addressing certain complications accompanied with, hydrophilic or hydrophobic drugs. GCPQ could be employed for targeted delivery and also for addressing the issues associated with non-chemotherapeutic drug moieties.

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