

Development of Ionic-Liquid Mediated Novel Drug Delivery System for Cancer Treatment

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Development of Ionic-Liquid Mediated Novel Drug Delivery System for Cancer Treatment

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CHAPTER-1

GENERAL INTRODUCTION

1.1 Ionic liquids (ILs) and their potential applications in drug delivery system (DDS)

1.1.1 What are ILs?

ILs are an interesting class of tunable and designer solvents with extraordinary properties over conventional organic solvents. ILs are organic salts that are typically liquid below 100 °C.¹ Some ILs are classified as room temperature ILs (RTILs) as they exist in liquid at ambient temperature.² ILs possess many unique and astonishing physico-chemical characteristics such as excellent thermal and chemical stability, extremely low vapor pressure, low flammability, nonvolatility, high ionic conductivity, recyclability, and multiple solvation abilities with organic and inorganic molecules.^{3,4} ILs are composed of an entire set of ions. Generally, an IL consists of cations of bulky asymmetric organic molecules such as alkylimidazolium $[R_1R_2IM]^+$, alkylpyridium $[RPy]^+$, tetraalkylammonium $[NR_4]^+$, tetraalkyl-phosphonium $[PR_4]^+$ or cholinium $[OH-R_1NR_3]^+$ and anions ($[Cl]^-$, $[NO_3]^-$, $[CH_3SOO]^-$, $[BF_4]^-$, $[CF_3(CF_2)_3COO]^-$, $[CH_3COO]^-$, $[(CF_3SOO)_2N]^-$, $[CF_3COO]^-$ or $[PF_6]^-$).⁵⁻⁷ It is so interesting that the pairing of thousands of organic cations and inorganic/organic anions can produce millions of ILs.

The field of ILs started with Humphrey Davy's pioneering work on the electrolytic decomposition of the simple molten salts under the influence of an applied dc electric field to yield the elements that initially chemically combined in the salt.^{1,8} The high melting simple salts were the primary works of Davy's study and his collected major work placed the investigation of the ionic liquids at the very beginning of organized chemical sciences.⁹ The Nobel Prize-winning physicist, Ramsay, was the first person who consciously used an ambient temperature ionic liquid for scientific purpose that was reported as "syrupy ionic liquids" a combination of acids and picoline.¹ The ILs are classified as aprotic, protic, inorganic and solvate ILs according to the book "Electrochemical Aspects of Ionic Liquids" edited by prof. Ohno.¹

1.1.2 Important Physicochemical Properties of ILs

The ILs are considered to be a potential candidate for different applications because of having extra-ordinary physicochemical characteristics over conventional organic solvents. But their physical and chemical properties can be finely tuned for a range of applications by simply alternating the cations or anions.¹⁰ The use of ILs as solvents for catalytic synthesis is one of

the potential application. Many favorable properties such as good solvents for a wide range of inorganic, organic and polymeric materials, adjustable polarity, and catalytic effects of ILs have been reported earlier, that facilitated the further investigations as reaction media in many organic and organometallic synthesis such as Diels-Alder reactions, Heck reactions, Friedel-Crafts reaction, alkylation, hydrogenation, hydroformylation, dimerization of butadiene, benzoic condensation, acylative cleavage of cyclic and acyclic ethers.^{1,2,10-16} ILs also have employed in the studies of enzymatic systems, such as lipase catalyzed kinetic resolution of 1-phenylethanol in ILs, enzymatic catalysis of formation of Z-aspartame in ILs, catalysis alcoholysis, ammoniolysis and perhydrolysis reaction by lipase in ILs.^{10,17,18} Nevertheless, ILs have been extensively used in variously application including biocatalysis, biomolecules purification, bio fuel production, energy conversion, anti-microbial agents, pharmaceutical drug delivery, drug formulation and so on.^{7,8,19,20} ILs have unusual physicochemical properties including excellent thermal and chemical stability, negligible vapor pressure, low flammability, non-volatility, high ionic conductivity, recyclability, and multiple solvation abilities with organic and inorganic compounds.^{3,10,11,21,22} The influence of the physicochemical characteristics on ILs behaviors is briefly discussed below-

❖ **Thermal Stability**

One of the important parameters of ILs is the possession of excellent thermal stability after conversion to ILs from their origin (cations or anions). Many biologically active compounds (active pharmaceutical ingredient, API) have been reported thermal unstable, but become stable upon formation of ILs.⁸ The formation of ILs is also known as API-IL showed great achievement for pharmaceutical application.^{6,20} It has been well established that protic ILs with proton transfer energies decompose before reaching their boiling point and the decomposition temperature varies within 100° C to 360° C. However, alteration of cation/anion with a bulky alkyl/heterocyclic chain can increase the decomposition temperatures with more thermally stable ILs.

❖ **Viscosity:**

Viscosity is known as vital properties of ILs for different applications. It is depend on the van der Waals interactions and hydrogen bonding capacity of a material. In case of ILs, anion's structure was believed to have a significant effect on the viscosity, while cation showed minor effect. It has been reported that, presence of a specific structure in anion could increase the viscosity significantly such as aromatic ring.^{5,23,24} If the ring number in cation could increase, it showed the increase of viscosity. It may be due to the increase of cation-anion interactions.²⁴

The behaviors of viscosity in ILs with altering the cation/anion remain interesting that need further substantiation with theoretical modelling.

❖ **Conductivity:**

The ionic conductivity of ILs depends on the viscosity as well as the molecular weight and the size of the ions. If the mobility of ions decreases that will cause to low or limited conductivity. Therefore, less ionic interaction and more delocalized charge will lead higher conductivity. If the size of cation increases, there may be possibilities in decrease of conductivity of ILs. It has been reported that having heterocyclic or small molecular weight structure in cation showed high ionic conductivity in ILs.^{6,15,24} However, no obvious relation can be found for the anions used in the system.

❖ **Density:**

Both cation and anion have influences on the density of ILs. In general, the density of an IL inversely proportional to the bulkiness of the cation grows. The reason may be due to the poor crystal packing of the ILs formed between the bulky cation and the weakly complex symmetrical anion. In another case, the change of anion such as replacing the hydrogen atom with heavier elements (F, Cl, Br) increases the density of ILs. It has been reported that the temperature has significant effect on the density of ILs, as the temperature changes from 293 to 313 K, the density decrease linearly as the temperature increases.^{19,24,25}

❖ **Melting Point**

The melting point is a fundamental physical property of molecules that have been used as unique identification. The melting point of ILs is considered to be significant because ILs have a large liquidus range determined by their low melting point as well as high decomposition points.¹¹ Most importantly, the solubility of ILs in aqueous and organic solvents is considered to be strongly correlated with their melting points.³ It has been reported that the freezing point occurs at the same temperature as the melting point.^{3,10,11} However, the thermal decomposition is strongly dependent on the structure of ILs. Generally, the imidazolium cations tend to be thermally more stable than the tetra-alkyl ammonium cations. High thermal stability can be provided by certain kinds of anions such as TFSI⁻(a31,[(CF₃SO₂)₂N]) and BETI⁻(a33, [C₂F₅SO₂]₂N). The relative anion stabilities are PF₆⁻>BETI⁻>TFSI⁻>CF₃SO₃⁻>BF₄⁻>Me[(CF₃SO₂)₃C]⁻>I, Br, Cl.^{6-8,11,20}

1.1.3 Different Applications of ILs

Over the last few decades, ILs have been continued to be used in different fields because of their tailored made properties. A list of fields of ILs application has been given below, but not all such as-

- ❖ ILs as a liquid lubricant in a diverse range of systems have found that these materials can show remarkable protection against wear and significantly reduce friction in the neat state. ILs can also be incorporated into non-polar base oils, replacing traditional anti-wear additives, with an excellent performance of the neat IL being maintained.
- ❖ ILs in analytical chemistry such as sample preparation, chromatographic/capillary electrophoretic (CE) separation and detection.
- ❖ ILs as electrolytes in aluminium electroplating, lithium batteries and in electrochemical capacitors.
- ❖ ILs as green solvent for lignin or lignocellulosic biomass deconstruction.
- ❖ ILs or IL-based polymer electrolytes in batteries.
- ❖ ILs for extraction and separation of bioactive compounds from plants.
- ❖ ILs as novel reaction media may offer a convenient solution to both the solvent emission and the catalyst recycling problem.
- ❖ ILs as solvents for polymerization processes.
- ❖ ILs in electrochemical sensing systems
- ❖ ILs for energy, materials and medicine.
- ❖ ILs as extracting agents for heavy metals.
- ❖ ILs for synthesis of inorganic nanomaterials.
- ❖ ILs as plasticizers for poly(methyl methacrylate).
- ❖ ILs as drug delivery system (DDS)

As our research is focusing on the application of ILs in DDS, we will further concentrate on this point.

1.1.4 Applications of ILs in DDS

ILs are reported as remarkable chemical compounds with multiple applications in many areas of modern science. Because of their highly tunable nature and extra ordinary physicochemical properties, ILs become essential components in the fields of synthesis, catalysis, extraction, electrochemistry, analytics and biotechnology.²⁶ The biological activities of ILs and their application in pharmaceuticals and medicine are newly emerging. It has been well reported that the pharmaceutical industries are facing a series of challenges in the delivery of many newly developed drug molecules because of their low, solubility, bioavailability, stability and polymeric conversion.¹⁹ These limitations are further exacerbated when drug molecules are insoluble or sparingly soluble in water and most pharmaceutically accepted solvents. Most importantly, sparingly soluble drugs lead to poor drug absorption and bioavailability via all method of drug administration, including oral delivery.²⁷ The oral delivery of many drug compounds hampered because of their water insolubility and/or lipophilicity such as tamoxifen, paclitaxel, docetaxel etc.¹ Moreover, many substrates that used for the synthesis of important drugs such as nucleoside analogs are poorly soluble in conventional solvents. It was reported as a prime concerning issue in the pharmaceutical industries.^{15,21,28–30} The use of ILs as solvents, co-solvents and/or formation of API-IL reported as an alternative way to solve the issue. The potential application of ILs was initially showed as their toxicity and antimicrobial activity in pharmaceuticals. Later, it was explored the use of ILs as greener candidates in biological applications such as enzymatic processes, whole-cell biocatalysis and protein stabilization.^{31–35} In the last few years, ILs have been grown as a potential candidate in different pharmaceutical fields including the formation of API-ILs, the use of ILs/IL mixture as solvents or co-solvents for the synthesis and purification of pharmaceuticals compounds and for poorly soluble drug molecules, and drug delivery systems.

1.1.5 ILs are Considered as Alternative of Conventional Organic Solvents

There are a lot of therapeutically active drug molecules in solid and crystalline forms that are poorly soluble in conventional organic solvents.³⁰ Their formations are very challenging for the formulation scientists. Furthermore, the low solubility of these molecules usually associated with the poor absorption and bioavailability upon oral administration. It is well reported in pharmaceutical industries and in modern medicine that, many promising drugs that are sparingly soluble in conventional solvents never enter a formulation due to their poor solubility as well as difficulties in delivery.^{6,20} One of the most easy way to overcome the problems and increase their solubility is to use of excipients as polar organic solvents, such as pyridine, N,N-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO).^{6,36} These solvents

are not inert vehicle. They have many drawbacks such as they are volatile, flammable and toxic. Even they are used as reaction media for synthesis of drugs; they must be removed from the final products.^{37,38} Their recovery and reuse steps are also difficult. Considering the extraordinary properties of ILs, it is highly desirable for ILs to be the potential alternative of conventional organic solvents.^{7,8,20} In fact, due to their tailor-made properties, ILs can dissolve complex molecules including biologically active compounds such as protein, nucleotides and amino acids under milder conditions. The tunable properties of ILs allow the possibilities of tailoring pharmaceutical solvents or use them in the synthesis for new drug forms with specifically desired properties that are effectively limited when water or molecular organic solvents are used.

1.1.6 Biocompatibility and Biodegradability of ILs

Because of the toxicological issue, the application of ILs as green solvents remain debatable. Before going to use of ILs in pharmaceutical application, it is important to confirm the biocompatibility of the molecules. It has been reported that the biologicals properties of ILs can be finely tuned simply by changing their cation/anion combination. It is possible to synthesize non-toxic ILs using the cation/anion from biological sources^{6,39} Biocompatible organic cation and inorganic anion may be an option to generate biocompatible ILs. Some studies showed that [bmim][Cl] on marine algae, or enzymes like acetyl cholinesterase was not acutely toxic ($EC_{50} \cong 13 \mu M$).^{40,41} In another study, imidazolium based ILs showed low/negligible toxicity towards Caco-2 cells.⁴¹ It has been reported and widely accepted that the biocompatibility/toxicity of any ionic liquids is largely depends on cation; of course anion also has role.⁴² For example, ionic liquids containing ammonium and alicyclic cations (morpholinium, piperidinium and pyrrolidinium) display lower toxicity than those with imidazolium and pyridinium.¹¹ Introduction of polar hydroxyl group/ether/nitrile functional group into the alkyl chain can significantly reduce the toxicity.⁶⁻⁸ Recently, ILs consist of cholinium/choline as the cation which incorporating a polar hydroxyl group considered as the most biocompatible and biodegradable one due to the use of cholinium/choline as an essential micronutrient that can be metabolism completely under aerobic conditions. The most desired anion should be amino acids that are the most abundant classes of organic compounds in nature well known as nontoxic, biodegradable and biocompatible.^{29,43} It has been reported that many useful and necessary chemicals are toxic, many pharmaceutically approved excipients such as DMSO and nonionic surfactants (e.g., Tween-80, T-80) display similar toxicities as observed from ILs.⁶ That's why, special care should be paid in selection of IL for DDS, because the toxicity of ILs depends significantly on their cation and the alkyl side chain.

1.1.7 ILs Strategies in DDS

Successful utilization of ILs in DDS can overcome the potential problems such as polymorphism, solubility and bioavailability of a molecule. In ILs, all ions can independently and/or simultaneously introduce a specific functionality or property adding tenability or flexibility to the design of new functional materials. Specific physiological and biological properties of ILs can be modified by changing the ion. There are essentially unlimited options for design and flexibility of ILs platform for drug delivery made strides within this field through several strategies of implementation including the following:

❖ API-ILs as Pharmaceuticals:

It is a strategy to prepare single or dual-active liquids salts of APIs or by liquefaction utilizing oligomeric ions, or by formation of liquid co-crystals. Prior to the synthesis of API-ILs, the approach of creating a liquid salt from biologically active ions (not yet APIs at this point) and a counter ion, resulted in the synthesis of room temperature ILs from the GRAS list (Generally Regarded as Safe) compounds, such as acesulfamate ([Ace]-), an artificial sweetener, paired with quaternary ammonium compounds (QACs).^{6,8} Here the use of QACs (Quaternary Ammonium Cations) and sweetener anions were prepared only for their properties as IL solvents prepared from food grade ions, and not for the biological properties of the ions. The first API-IL was ranitidine-docusate, where the liquification of ranitidine was occurred due the IL conversion with docusate.⁷ It was also reported as the polymorphic conversion, as the ranitidine docusate was a dark red liquid with glass transition temperature T_{glass} of -12 °C. Similarly, by combining lidocaine hydrochloride and sodium docusate, lidocaine docusate was prepared, resulted API-IL with T_{glass} of -29°C. Using the same strategy, the API-IL of didecyldimethylammonium ibuprofenate from sodium ibuprofen and didecyldimethylammonium bromide was prepared, where sodium ibuprofen has anti-inflammatory and didecyldimethylammonium bromide has antibacterial and anti-inflammatory properties. The resulting API-IL showed both of cationic and anionic properties and the active ion could be either cation or anion or both.^{6,8,11,20} Not only APIs could be converted into pure liquids, but also new biological functions and modes of delivery were considered achievable. In fact, the counter ion in liquid salts can be used not only for preventing crystallization, but also for its action as the second active component allowing for dual-active performance, similar to the concept of using both ions in an IL to serve different tasks.^{23,44–46} In theory, any combination of two or more drugs is possible as long as both APIs form stable ions. The judicious selection of appropriate active ions not only allows for dual-functional performance,

but possible synergistic effects, introducing new therapeutic properties not inherent in the pure neutral or known salt forms.

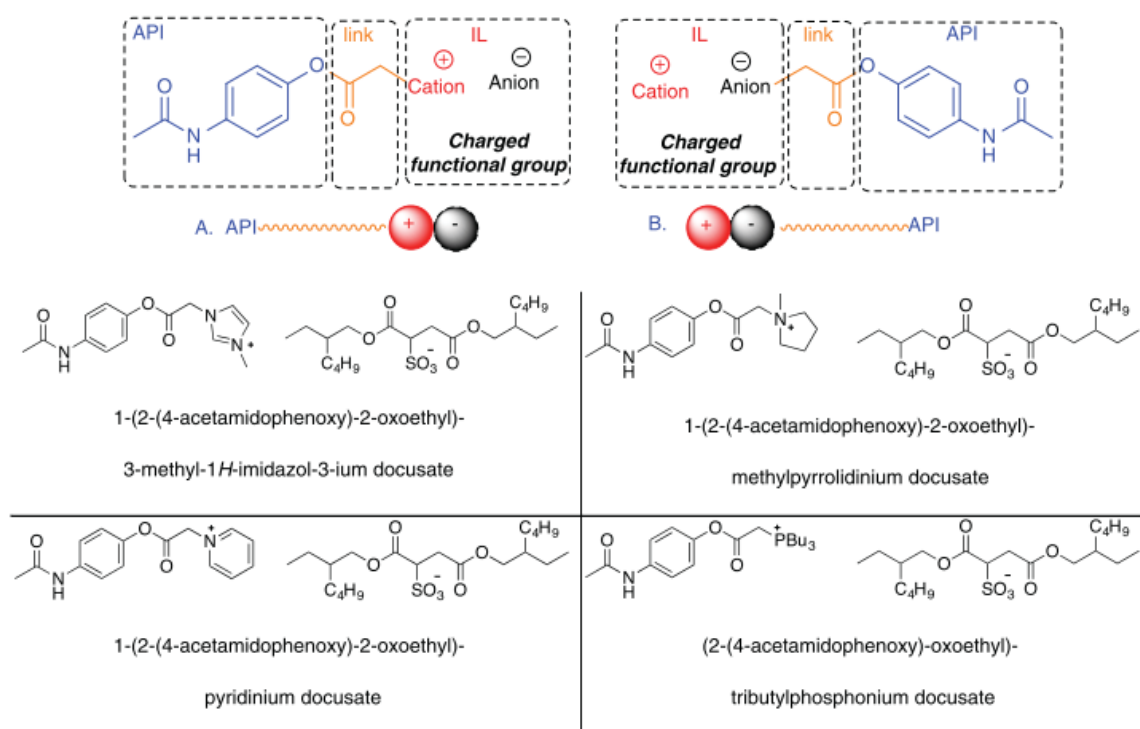


Figure 1-1. API-IL prodrug strategy (top) and compounds of type A synthesized in a proof-of-concept study. Reprint with permission from ref.20.

❖ Formation of Prodrug API-ILs:

This is another potential strategy for formation of prodrug API-ILs where one of the ions of API-ILs in DDS. Prodrugs are known as pharmaceutically inactive compounds that are converted into the active drug through a metabolic biotransformation within the body. Upon conversion into prodrugs, some advantages such as enhanced solubility, targeted site specificity, prevention from rapid metabolic degradation, extended blood circulation and reduced toxicity of drugs reported to be attained before reaching to the site of action.^{8,47,48} It has been reported that prodrugs may suffer from the same issue as solid drugs (e.g., polymorphism). Using the API-IL strategy in a prodrug approach, advantages such as removal of unwanted properties of a solid drug along with addition of the prodrug properties can be achieved. It was done by adding a hydrolyzable functional group to the neutral API, forming charge or ionizable, which then can be combined with appropriate counterions leading to new ILs. It is also possible to provide the second functional group as the second active molecule in the prodrug strategy. The prodrug API-ILs can be easily prepared from non- or difficult to ionize APIs using functionalization of the API with a hydrolysable charged group. Fig. 1-1 shows the API-IL

prodrug strategy for the formation of pyridinium docusate and tributylphosphonium docusate API-IL prodrugs.^{20,49}

❖ ILs as Excellent Solubilizing Agents

This is one of the great strategies for solubilization of poorly water-soluble drugs using ILs as solubilizing agents. Firstly, ILs can work as a surfactant and APIs were entrapped/solubilized within micelles, which could work as a reservoir for the control release. Secondly, IL-assisted non-aqueous microemulsions that are stabilized by an addition of external surfactants and thirdly, the design of ILs as hydrophilic-lipophilic balanced (HLB) hydrotropes that keep the APIs dissolved once added to water.

It has been reported that a number of drug molecules showed resistant to dissolution in water and common organic solvents. They are known as poorly water soluble drugs and they have low dissolution rate in the gastrointestinal (GI) tract with very low bioavailability. Fortunately, ILs are found to be very effective in dissolving some important drug molecules that are sparingly soluble in water and most pharmaceutically accepted solvents.^{7,24,45,49,50} While studying in the solvation capacity of ILs, it was observed that the IL cation has very little effect on the solvation capacity, whereas the anion plays the most significant role in the solubility for the drug molecules. The hydrophilic ILs with coordinating anions such as $[\text{CH}_3\text{COO}]^-$ or $[\text{MePhos}]^-$ were found to be more effective and able to dissolve significant amounts of drug compounds at room temperature. Surprisingly, it was observed that hydrophobic ILs with non-coordinating anions such as $[\text{BF}_4]^-$, $[\text{PF}_6]^-$ and $[\text{NTf}_2]^-$ could not dissolve such drug molecules. The solubility of other sparingly soluble substances such as cellulose in ILs also showed a similar trend. By combining the results, it was quite clear that the dissolution of drugs in ILs involves the formation of hydrogen bonds between the IL anions and the drug molecules.

It has been reported that the mechanism of drug solubility in ILs depends on the molecular forces. The capacity of weaken or break of inter and intramolecular hydrogen bonding can be designed by selecting the cations and anions. Special emphasis should be given to ILs on water retain, so that the ILs-loaded drugs could be suitable for the practical drug delivery system. A study on solubility of a poorly water-soluble cardioactive prototype drug, 3,4-methylenedioxybenzoyl-2-thienylhydrazone, in various imidazolium-based ILs, denoted $[\text{C2mim}][\text{MePhos}]$, $[\text{C2mim}][\text{EtPhos}]$, $[\text{C2mim}][\text{CH}_3\text{COO}]$, $[\text{C1mim}][\text{MePhos}]$, $[\text{C4mim}][\text{CH}_3\text{COO}]$, $[\text{C4mim}][\text{BF}_4]$ and $[\text{C4mim}][\text{NTf}_2]$, revealed that ILs containing a $[\text{CH}_3\text{COO}]$ anion was attributed to the good capacity of this anion to form hydrogen bonds and delocalize the charges between the oxygen atoms.^{7,37} In another study, increased solubility of

glibenclamide (an antidiabetic drug) in the IL [Choline][Trp] due to the formation of complex hydrogen bonds and π - π interactions among the aromatic rings was reported.⁵¹

In some studies, the hydrophobicity was claimed to be responsible for effective solubility, where the [P_{6,6,6,14}][NTf₂] IL used for high solubilization of 4 iso-butylacetophenone, thymoquinone, coumarin and ibuprofen. In that study, N-acetyl-L-cysteine was found to be less soluble as like as isoniazide, 4-hydroxycoumarin and pyrazinecarboxamide.⁵² Using imidazolium-based ILs with [NTf₂] and [OTf] as anions showed that the hydrophilic solutes N-acetyl-L-cystein and 4-hydroxycoumarin were highly soluble in [C₂mim][OTf] IL. The [C₁₀mim][NTf₂] IL was found to be best for coumarin.^{7,38,48}

It has been reported that the alkyl side chain linked to anion/cation has significant effect on solubility in ILs. While using the [C₂mim] as the cation, combined with methyl phosphate and ethyl phosphate as anions, enhanced solubility of drugs were achieved for longer side chain compared to smaller of anions.⁵³ Surprisingly, the cation showed the opposite trends such as the solubilities of N-acetyl-L- cysteine and 4-hydroxycoumarin obtained in [NTf₂]-containing ILs were found to decrease with the increase of the alkyl chain length in the IL cation. But the reverse trend was obtained for coumarin.^{38,48} For lipid soluble drugs such as danazol (DNZ) and itraconazole (ITCZ), the solubility was found to be increased with the increase in the alkyl chain length of the pyridinium cation. While the same cation was combined with a more hydrophilic anion, the solubility was enhanced significant for both of DNZ and ITCZ.⁵⁴ In another study, pyrazine-2-carboxamide was found to achieve lower solubilization in ILs containing [NTf₂] compared to [OTf]. The [C₁₀mim][OTf] was claimed to be responsible for the best solvent for carboxamide drug.⁵⁵ The solubility of isoniazid and pyrazinecarboxamide was examined in a series of ammonium-based ILs such as [DDA][NO₃], [BA][NO₃] and [EDMA][NTf₂]. [DDA][NO₃] and found that the solubility of isoniazid was higher than pyrazinecarboxamide. The reason was claimed to the interactions between the cations and the drug molecule. The effective dissolution of the drug was depended on the ability of the solvent to form interactions with the drugs that are more powerful than the interactions of solute-solute and solvent –solvent.⁵³

One more important factor, temperature, was also considered to have effective role in drugs dissolution in ILs. In a study, the solubilities of ibuprofen (IBU) and paracetamol (PCM) were checked in [C₄mim][PF₆]- and [C₆mim][PF₆]-based ILs at different temperatures and found the solubility of both drug increased with temperature in both ILs.⁴ While examining the solubility of erythromycin in ILs, it showed the solubility enhanced significantly by an increase in the temperature and as well as the alkyl chain length in cations.⁵⁶

Therefore, the ability of many ILs to enhance the solubility and permeating ability of poorly water-soluble drugs can be a potential strategy of improved absorption and bioavailability of the drugs at the targeted site of action.

1.2 Cancer and Obstacles of Cancer Treatment

1.2.1 Cancer and its Treatment

❖ Cancer

Cancer is a major public health problem not only in United States but also in all over the world. It is a disease condition that caused by the persistence tissue injury and interaction between host-environment. It can lead to a various genetic (mutation), epigenetic (loss of heterozygosity) and global transcriptome changes due to repeated exposure of carcinogens such as tobacco, ultraviolet light and infections.⁵⁷⁻⁶⁰ Over the last decade, it has been a great challenge to the healthcare professionals and scientists to control the increased occurrence of cancer and worldwide prevalence. It has been reported that the global cancer death could be increased about 45% within 2030 by WHO statistics and of them 70% would be contributed by developing countries.⁶¹

By the way, cancer is not a single condition but a complex and collection of disease, which can arise in any tissue in our body. In our body, normal cells are monitored by a myriad of mechanism that keep them working in cooperation with other cells. Every cancer starts as a disruption of the normal activity of cells such as they divide and move whenever and whatever they like and attract blood vessels to themselves disobeying the signals.^{59,62} Each cancer has its own unique pattern of bad behavior according to the tissue where they formed, the mutations the cells have adopted and the chemistry in an individual's body system. Even each cancer is different, there are a shared set of behaviors that characterizes all cancer cells such as:

❖ Uncontrolled Growth:

Normal cells grow and divide when they get messages from the cells around them that it's time to do so. Cancer cells, on the other hand, are able to jump-start growth by themselves and therefore can divide and make new copies of them independent of what's going on in the cells around them. Lack of response to stop signals: A healthy cell will stop dividing when one of two things happens: it receives signals from nearby cells that the "neighborhood" is crowded enough, or its growth cellular machinery is damaged. Ordinarily, a cell will take time out to repair problems. Cancer cells just keep on going, proliferating under conditions that would stop normal cells and making new copies of cells with damaged DNA.⁶⁰

❖ **Immortality:**

Almost all the cells in your body are programmed to stop functioning or commit suicide when cellular machinery is damaged beyond repair, when they're infected by a virus, when there are too many cells, or when cellular functions begin to break down. An aging cell may simply stop dividing, or it may undergo a sequence of events called programmed cell death or apoptosis. Cancer cells ignore these stop signals and are thus able to expand their numbers.^{60,63}

❖ **Ability to Divide Infinity:**

Healthy cells eventually stop dividing but continue to live. Their growth is stopped by the fact that their DNA is programmed to tolerate a certain number of replications and sends a "stop" signal when it's reached its limit. Cancer cells evolve new ways to evade these signals. As a result, they continue to replicate, producing new cells that have miscopied or mutated DNA strands.^{58,64}

❖ **Recruiting a Food Supply:**

All cells need to be fed by oxygen and nutrients in the blood. Ordinarily, the body's systems carefully regulate the growth of new feeder blood vessels, a process called angiogenesis. A tumor of cancerous cells typically skirts these systems and independently signals the body to feed it, causing new vessels to grow into the tumor. This vascularization of a tumor marks one of the defining moments when a precancerous tissue crosses the line and becomes a true cancer.⁵⁹

❖ **Random Migration**

In healthy tissues, the cells stay different, there is a shared set of behavior where they are, adhering to each other in structures that characterize the tissue and assist in its function. Mature cancer cells, in contrast, can let go of these molecular handholds and relocate to other parts of the body. This cell movement, called metastasis, is one of the most defining characteristics of malignance. The resulting eruption of tumors in distant parts of the body is what brings about the majority of deaths from cancer.^{65,66} Taken together, these rogue qualities are considered to be the hallmarks of cancer. Precancerous cells may show uncontrolled growth and the lack appropriate response to stop signals and so they may divide repeatedly. Each new division is subject to frequent genetic errors. When such cells proliferate, they continue to pass along their disruptive mutations and likely acquire new ones, eventually creating a cancerous tumor. Tumor cells often acquire the first five characteristics before they move out to other parts of the body. This helps explain why catching a cancer early is important: If you detect it before infected by a virus, when there are it has acquired all the hallmarks, you can prevent the metastasis.

❖ **Treatments of Cancer:**

Anticancer therapy is one of the options for the cancer treatment along with the surgical treatment and radiation therapy. In general, anticancer drugs are divided into three categories: cytotoxic, biological and hormonal agents. Cytotoxic agents are the traditional therapies that damage cancer cells by interfering with DNA or its precursor, inhibit the cellular division. However, this kind of agents has the great drawback of killing healthy cells along with cancer cells.^{61,64} A major types of cytotoxic agents are alkylating agents, antimetabolites and plant alkaloid.^{60,64,67,68} There have been reported that the biological agents or targeted agents are included monoclonal antibodies, and cancer vaccine which is known as an immune therapy used to boost up body's immune systems. The hormonal therapy directly focuses on hormone-dependent pathways that associate with the development of cancer cells.^{66,69,70}

Now a day, most anticancer drugs are administered intravenously because it is the most direct route to overcome the variable absorption patterns of the gastrointestinal tract. The intravenous route leads to immediate and complete bioavailability and accurate dosing regiments. The preparation of injectable formulation requires the use of specific pharmaceutically excipients. The toxicological effects of the excipients in the final formulation are very concerning issue before therapeutic application. Sometime, premedication is required to reduce the effects of excipients, which can lead additional problems. However, the effective selection of excipients considered the biocompatibility may reduce the side effects associated with excipients during anticancer therapies.

1.2.2 Major Obstacles of Anti-Cancer Drugs and Treatment

It has been reported that major progress achieved in cancer treatment results from conventional drugs used for cytotoxic therapy.⁵⁸ However, most of anticancer drugs are water-insoluble, delaying their clinical effectiveness.^{58,64} Moreover, a number of anticancer drugs such as camptothecin, paclitaxel and etoposide are lipophilic, polar and unstable in aqueous media and undergo less potent decomposition through hydrolysis. These characteristics make their design of formulation difficult.^{60,65,71}

From the physicochemical point of view, major anticancer drugs are sparingly water-soluble that make them difficult to make an effective formulation. The pharmaceutical formulation could be possible if the solubility could be improved of those drugs.⁶¹ The use of concerning pharmaceutical excipients is well known for improved solubilization of anticancer drugs using water miscible solvents/co-solvents such as ethanol, methanol, methyl chloride or acetonitrile, 14 mM in isopropanol and water-insoluble organic solvents such as oils,

triglycerides, vitamin E, or their associations.^{65–68,72,73} Formation of micelles, liposomes, micro and nanocapsules, dendrimers, emulsions, micro/nano emulsions were largely used to increase apparent water solubility of anticancer drugs.^{58,61,64,68,71,73,74}

1.3 Aim and Outline of this Thesis

Now it is widely reported that ILs can act as superior solvent systems and/or a delivery technology to relief the growing pressure on pharmaceuticals industries. In the last few years, room temperature ILs have been increasingly exploited as solvents and/or (co)solvents and/or reagents in a wide range of pharmaceutical applications due to their tailor-made chemical, physical and biological properties. Therefore, the aim of this research is to search ILs that are nontoxic and biodegradable for the development of novel drug delivery systems for sparingly water-soluble anticancer drugs. In particular, the biocompatibility and the capability of ILs to dissolve sparingly water-soluble drugs are investigated and the cellular *in-vitro* & *in-vivo* studies are carried out to evaluate their potential application as effective drug delivery systems.

In **chapter-2**, we report a new paclitaxel formulation with a mixture of biocompatible IL, Tween-80 (polyoxyethylene 20 sorbitan monooleate), ethanol, and H₂O as a delivery system in a specific ratio, where ILs enhance the solubility and stability of paclitaxel in the formulation instead of cremophor EL (CrEL). Furthermore, the IL-mediated paclitaxel formulation exhibits small particle size and a narrow size distribution, lower cytotoxicity, and similar anti-tumor activity to CrEL-mediated paclitaxel (e.g., Taxol) against HeLa cells in *in-vitro* experiments. Moreover, the hypersensitivity effects of the IL-based formulation are found to be significantly reduced compared with Taxol in a human leukemia cell line, THP-1.

In **chapter-3**, as a follow-up to previous study, further investigation of the IL-based paclitaxel formulation is reported where *in vivo* biocompatibility, pharmacokinetics, antitumor activity, and hypersensitivity are evaluated using a mouse model with CrEL-mediated paclitaxel (e.g., Taxol) as a reference.

In **chapter-4**, we report a one-step emulsification and rapid freeze-drying process to develop a curcumin–ionic liquid (CCM–IL) complex that could be readily dispersed in water with a significantly enhanced solubility and stability (half-life) compared to that of free CCM. For this study, the IL consists of choline as cation and oleic acid as the anion used as a surfactant. Both the cation (choline) and anion (oleic acid) in the IL are widely accepted as a biocompatible one. Furthermore, oleic acid has been reported to have many interesting biological functions including anti-inflammatory, anti-carcinogenic, anti-oxidative, and immune system activation

properties. Therefore, combining the therapeutic effects of the surfactant and drug considered as a novel approach for the preparation of surfactant–drug complexes.

Finally, in **chapter-5**, we summarized the findings of this research and briefly discussed further research directions using ILs related to this research.

References:

- (1) Austen Angell, C.; Ansari, Y.; Zhao, Z. Ionic Liquids: Past, Present and Future. *Faraday Discuss.* **2012**, *154* (January), 9–27.
- (2) Welton, T. Room-Temperature Ionic Liquids. Solvents for Synthesis and Catalysis. *Chem. Rev.* **1999**, *99* (8), 2071–2084.
- (3) Ghandi, K. A Review of Ionic Liquids, Their Limits and Applications. *Green Sustain. Chem.* **2014**, *04* (01), 44–53.
- (4) Dos Santos, A. D.; Morais, A. R. C.; Melo, C.; Bogel-Lukasik, R.; Bogel-Lukasik, E. Solubility of Pharmaceutical Compounds in Ionic Liquids. *Fluid Phase Equilib.* **2013**, *356*, 18–29.
- (5) Moniruzzaman, M.; Tamura, M.; Tahara, Y.; Kamiya, N.; Goto, M. Ionic Liquid-in-Oil Microemulsion as a Potential Carrier of Sparingly Soluble Drug: Characterization and Cytotoxicity Evaluation. *Int. J. Pharm.* **2010**, *400* (1–2), 243–250.
- (6) Moniruzzaman, M.; Goto, M. Ionic Liquids: Future Solvents and Reagent for Pharmaceuticals. *J. Chem. Eng. Japan* **2011**, *44* (6), 370–381.
- (7) Adawiyah, N.; Moniruzzaman, M.; Hawatulaila, S.; Goto, M. Ionic Liquids as a Potential Tool for Drug Delivery Systems. *MedChemComm.* The Royal Society of Chemistry, 2016, pp 1881–1897.
- (8) Marrucho, I. M.; Branco, L. C.; Rebelo, L. P. N. Ionic Liquids in Pharmaceutical Applications. *Annu. Rev. Chem. Biomol. Eng.* **2014**, *5* (1), 527–546.
- (9) Davy, H. *Researches, Chemical and Philosophical, Chiefly Concerning Nitrous Oxide*; Printed for J. Johnson ... by Biggs and Cottle Bristol: London, 1800.
- (10) Zhao, H. Current Studies on Some Physical Properties of Ionic Liquids. *Phys. Chem. Liq.* **2003**, *41* (6), 545–557.
- (11) Zhang, S.; Sun, N.; He, X.; Lu, X.; Zhang, X. Physical Properties of Ionic Liquids: Database and Evaluation. *J. Phys. Chem. Ref. Data* **2006**, *35* (4), 1475–1517.
- (12) Akbik, D.; Ghadiri, M.; Chrzanowski, W.; Rohanizadeh, R. Curcumin as a Wound Healing Agent. *Life Sci.* **2014**, *116* (1), 1–7.
- (13) Hwu, J. R.; Lin, Y. S.; Josephrajan, T.; Hsu, M. H.; Cheng, F. Y.; Yeh, C. S.; Su, W. C.; Shieh, D. Bin. Targeted Paclitaxel by Conjugation to Iron Oxide and Gold Nanoparticles. *J. Am. Chem. Soc.* **2009**, *131* (1), 66–68.
- (14) Kim, S. Y.; Hwang, J. Y.; Seo, J. W.; Shin, U. S. Production of CNT-Taxol-Embedded PCL Microspheres Using an Ammonium-Based Room Temperature Ionic Liquid: As a

- Sustained Drug Delivery System. *J. Colloid Interface Sci.* **2015**, *442*, 147–153.
- (15) Wasserscheid, P.; Keim, W. Ionic Liquids—New “Solutions” for Transition Metal Catalysis. *Angew. Chemie* **2000**, *39* (21), 3772–3789.
 - (16) Tourné-Péteilh, C.; Coasne, B.; In, M.; Brevet, D.; Devoisselle, J. M.; Vioux, A.; Viau, L. Surfactant Behavior of Ionic Liquids Involving a Drug: From Molecular Interactions to Self-Assembly. *Langmuir* **2014**, *30* (5), 1229–1238.
 - (17) Sheldon, R. Catalytic Reactions in Ionic Liquids. *Chem. Commun.* **2001**, No. 23, 2399–2407.
 - (18) Erbdinger, M.; Mesiano, A. J.; Russell, A. J. Enzymatic Catalysis of Formation of Z-Aspartame in Ionic Liquid an Alternative to Enzymatic Catalysis in Organic Solvents. *Biotechnology Progress*. American Chemical Society (ACS) December 1, 2000, pp 1129–1131.
 - (19) Ghandi, K. A Review of Ionic Liquids, Their Limits and Applications. *Green Sustain. Chem.* **2014**, *04* (01), 44–53.
 - (20) Shamshina, J. L.; Barber, P. S.; Rogers, R. D. Ionic Liquids in Drug Delivery. *Expert Opin. Drug Deliv.* **2013**, *10* (10), 1367–1381.
 - (21) Ratti, R. Ionic Liquids: Synthesis and Applications in Catalysis. *Adv. Chem.* **2014**, *2014* (3), 1–16.
 - (22) Lei, Z.; Chen, B.; Koo, Y.-M.; MacFarlane, D. R. Introduction: Ionic Liquids. *Chem. Rev.* **2017**, *117* (10), 6633–6635.
 - (23) Seedher, N.; Kanojia, M. Micellar Solubilization of Some Poorly Soluble Antidiabetic Drugs: A Technical Note. *AAPS PharmSciTech* **2008**, *9* (2), 431–436.
 - (24) Abe, M.; Kuroda, K.; Sato, D.; Kunitamura, H.; Ohno, H. Effects of Polarity, Hydrophobicity, and Density of Ionic Liquids on Cellulose Solubility. *Phys. Chem. Chem. Phys.* **2015**, *17* (48), 32276–32282.
 - (25) Lu, B.; Xu, A.; Wang, J. Cation Does Matter: How Cationic Structure Affects the Dissolution of Cellulose in Ionic Liquids. *Green Chem.* **2014**, *16* (3), 1326–1335.
 - (26) Earle, M. J.; Seddon, K. R. Ionic Liquids. Green Solvents for the Future. *Pure Appl. Chem.* **2007**, *72* (7), 1391–1398.
 - (27) Roger, R. D.; Seddon, K. R. Ionic Liquids-Solvents of the Future? *Science (80-.)*. **2003**, *302*, 792–793.
 - (28) Zhang, S.; Sun, J.; Zhang, X.; Xin, J.; Miao, Q.; Wang, J. Ionic Liquid-Based Green Processes for Energy Production. *Chem. Soc. Rev.* **2014**, *43* (22), 7838–7869.
 - (29) Dobler, D.; Schmidts, T.; Klingenhöfer, I.; Runkel, F. Ionic Liquids as Ingredients in Topical Drug Delivery Systems. *Int. J. Pharm.* **2013**, *441* (1–2), 620–627.
 - (30) Kalepu, S.; Nekkanti, V. Insoluble Drug Delivery Strategies: Review of Recent Advances and Business Prospects. *Acta Pharm. Sin. B* **2015**, *5* (5), 442–453.
 - (31) Jin, W.; Yang, Q.; Zhang, Z.; Bao, Z.; Ren, Q.; Yang, Y.; Xing, H. Self-Assembly Induced Solubilization of Drug-like Molecules in Nanostructured Ionic Liquids. *Chem. Commun.* **2015**, *51* (67), 13170–13173.

- (32) Kärkkäinen, J. *Preparation and Characterization of Some Ionic Liquids and Their Use in the Dimerization Reaction of 2-Methylpropene*; 2007; Vol. A480.
- (33) Liu, Q.-P.; Hou, X.-D.; Li, N.; Zong, M.-H. Ionic Liquids from Renewable Biomaterials: Synthesis, Characterization and Application in the Pretreatment of Biomass. *Green Chem.* **2012**, 14 (2), 304–307.
- (34) Yang, Q.; Xu, D.; Zhang, J.; Zhu, Y.; Zhang, Z.; Qian, C.; Ren, Q.; Xing, H. Long-Chain Fatty Acid-Based Phosphonium Ionic Liquids with Strong Hydrogen-Bond Basicity and Good Lipophilicity: Synthesis, Characterization, and Application in Extraction. *ACS Sustain. Chem. Eng.* **2015**, 3 (2), 309–316.
- (35) Liu, C.-Z.; Wang, F.; Stiles, A. R.; Guo, C. Ionic Liquids for Biofuel Production: Opportunities and Challenges. *Appl. Energy* **2012**, 92, 406–414.
- (36) Sikarra, D.; Shukla, V.; Kharia, A. A.; Chatterjee, D. P. Techniques for Solubility Enhancement of Poorly Soluble Drugs: An Overview. *J. Med. Pharm. www.jmpas.com Allied Sci. J. Med. Pharm. Allied Sci. J. Med. Pharm. Allied Sci.* **2012**, 01 (01), 1–22.
- (37) dos Santos, A. D.; Morais, A. R. C.; Melo, C.; Bogel-Lukasik, R.; Bogel-Lukasik, E. Solubility of Pharmaceutical Compounds in Ionic Liquids. *Fluid Phase Equilib.* **2013**, 356 (5), 18–29.
- (38) dos Santos, A. D.; Morais, A. R. C.; Melo, C.; Bogel-Lukasik, R.; Bogel-Lukasik, E. Solubility of Pharmaceutical Compounds in Ionic Liquids. *Fluid Phase Equilib.* **2013**, 356, 18–29.
- (39) Pernak, J.; Chwala, P.; Syguda, A. Room Temperature Ionic Liquids — New Choline Derivatives. *ChemInform* **2004**, 35 (33), 2004.
- (40) Stolte, S.; Matzke, M.; Arning, J.; Bösch, A.; Pitner, W. R.; Welz-Biermann, U.; Jastorff, B.; Ranke, J. Effects of Different Head Groups and Functionalised Side Chains on the Aquatic Toxicity of Ionic Liquids. *Green Chem.* **2007**, 9 (11), 1170–1179.
- (41) Pernak, J.; Sobaszekiewicz, K.; Foksowicz-Flaczyk, J. Ionic Liquids with Symmetrical Dialkoxymethyl-Substituted Imidazolium Cations. *Chem. - A Eur. J.* **2004**, 10 (14), 3479–3485.
- (42) Patil, M. L.; Sasai, H. Recent Developments on Chiral Ionic Liquids: Design, Synthesis, and Applications. *Chem. Rec.* **2008**, 8 (2), 98–108.
- (43) Petkovic, M.; Ferguson, J. L.; Gunaratne, H. . N.; Ferreira, R.; Leitao, M. C.; Seddon, K. R.; Rebelo, L. P. N.; Pereira, C. S. Novel Biocompatible Cholinium-Based Ionic Liquids-Toxicity and Biodegradability. 2010.
- (44) Itoh, H.; Naka, K.; Chujo, Y. Synthesis of Gold Nanoparticles Modified with Ionic Liquid Based on the Imidazolium Cation. *J. Am. Chem. Soc.* **2004**, 126 (10), 3026–3027.
- (45) Tsuda, T.; Imanishi, A.; Torimoto, T.; Kuwabata, S. Nanoparticle Preparation in Room-Temperature Ionic Liquid under Vacuum Condition. In *Ionic Liquids: Theory, Properties, New Approaches*; 2011.
- (46) Mehnert, C. P.; Cook, R. a; Dispenziere, N. C.; Afeworki, M. Supported Ionic Liquid Catalysis--a New Concept for Homogeneous Hydroformylation Catalysis. *J. Am. Chem. Soc.* **2002**, 124 (44), 12932–12933.

- (47) Dobler, D.; Schmidts, T.; Klingenhöfer, I.; Runkel, F. Ionic Liquids as Ingredients in Topical Drug Delivery Systems. *Int. J. Pharm.* **2013**, *441* (1–2), 620–627.
- (48) Forte, A.; Melo, C. I.; Bogel-Lukasik, R.; Bogel-Lukasik, E. A Favourable Solubility of Isoniazid, an Antitubercular Antibiotic Drug, in Alternative Solvents. *Fluid Phase Equilib.* **2012**, *318*, 89–95.
- (49) Egorova, K. S.; Gordeev, E. G.; Ananikov, V. P.; Zelinsky, N. D. Biological Activity of Ionic Liquids and Their Application in Pharmaceutics and Medicine. *Chem. Rev.* **2017**, *117* (10), 7132–7189.
- (50) Moniruzzaman, M.; Kamiya, N.; Goto, M. Ionic Liquid Based Microemulsion with Pharmaceutically Accepted Components: Formulation and Potential Applications. *J. Colloid Interface Sci.* **2010**, *352* (1), 136–142.
- (51) Alawi, M. A.; Hamdan, I. I.; Sallam, Als. A.; Heshmeh, N. A. Solubility Enhancement of Glibenclamide in Choline-Tryptophan Ionic Liquid: Preparation, Characterization and Mechanism of Solubilization. *J. Mol. Liq.* **2015**, *212*, 629–634.
- (52) Faria, R. A.; da Ponte, M. N.; Bogel-Lukasik, E. Solubility Studies on the System of Trihexyl(Tetradecyl)Phosphonium Bis[(Trifluoromethyl)Sulfonyl]Amide) Ionic Liquid and Pharmaceutical and Bioactive Compounds. *Fluid Phase Equilib.* **2015**, *385*, 1–9.
- (53) Resende De Azevedo, J.; Letourneau, J. J.; Espitalier, F.; Ré, M. I. Solubility of a New Cardioactive Prototype Drug in Ionic Liquids. *J. Chem. Eng. Data* **2014**, *59* (6), 1766–1773.
- (54) Williams, H. D.; Sahbaz, Y.; Ford, L.; Nguyen, T. H.; Scammells, P. J.; Porter, C. J. H. Ionic Liquids Provide Unique Opportunities for Oral Drug Delivery: Structure Optimization and in Vivo Evidence of Utility. *Chem. Commun.* **2014**, *50* (14), 1688–1690.
- (55) Lourenço, C.; Melo, C. I.; Bogel-Lukasik, R.; Bogel-Lukasik, E. Solubility Advantage of Pyrazine-2-Carboxamide: Application of Alternative Solvents on the Way to the Future Pharmaceutical Development. *J. Chem. Eng. Data* **2012**, *57* (5), 1525–1533.
- (56) Manic, M. S.; Najdanovic-Visak, V. Solubility of Erythromycin in Ionic Liquids. *J. Chem. Thermodyn.* **2012**, *44* (1), 102–106.
- (57) Wang, Y.; Wu, K. C.; Zhao, B. X.; Zhao, X.; Wang, X.; Chen, S.; Nie, S. F.; Pan, W. S.; Zhang, X.; Zhang, Q. A Novel Paclitaxel Microemulsion Containing a Reduced Amount of Cremophor EL: Pharmacokinetics, Biodistribution, and In Vivo Antitumor Efficacy and Safety. *J. Biomed. Biotechnol.* **2011**, *2011*, 10.
- (58) Koide, H.; Asai, T.; Shimizu, K.; Oku, N. Drug Carrier Systems for Anticancer Agents. *Polym. Biomater. Med. Pharm. Appl. Vol. 2* **2013**, *67* (March), 153–170.
- (59) Srinivas, N. Formulation Development for Cancer Compounds - Biopharmaceutical Issues and Perspectives. *Asian J. Pharm.* **2009**, *3* (2), 90.
- (60) Thanki, K.; Gangwal, R. P.; Sangamwar, A. T.; Jain, S. Oral Delivery of Anticancer Drugs: Challenges and Opportunities. *Journal of Controlled Release*. 2013, pp 15–40.
- (61) Yadav, S. K.; Parvez, N.; Sharma, P. K. An Overview and New Strategies to Improve Formulation Development of Anticancer Drugs. *Adv. Biol. Res. (Rennes)*. **2014**, *8* (6), 233–243.

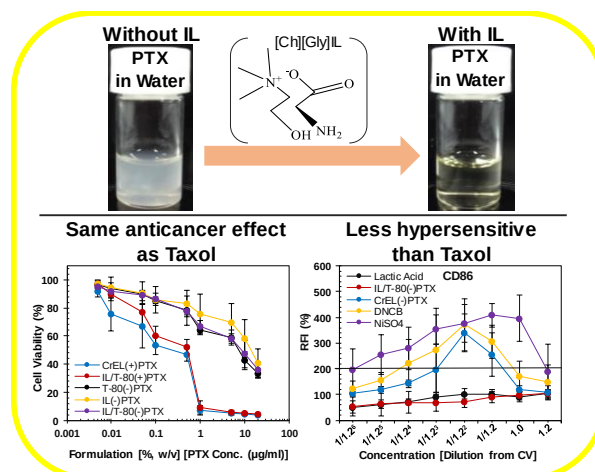
- (62) Cho, H.-Y.; Kang, J.-H.; Ngo, L.; Tran, P.; Lee, Y.-B. Preparation and Evaluation of Solid-Self-Emulsifying Drug Delivery System Containing Paclitaxel for Lymphatic Delivery. *J. Nanomater.* **2016**, *2016*, 1–14.
- (63) Willis, S. The Bcl-2-Regulated Apoptotic Pathway. *J. Cell Sci.* **2003**, *116* (20), 4053–4056.
- (64) Hare, J. I.; Lammers, T.; Ashford, M. B.; Puri, S.; Storm, G.; Barry, S. T. Challenges and Strategies in Anti-Cancer Nanomedicine Development: An Industry Perspective. *Adv. Drug Deliv. Rev.* **2017**, *108*, 25–38.
- (65) Ünal, H.; Öztürk, N.; Bilensoy, E. Formulation Development, Stability and Anticancer Efficacy of Core-Shell Cyclodextrin Nanocapsules for Oral Chemotherapy with Camptothecin. *Beilstein J. Org. Chem.* **2015**, *11*, 204–212.
- (66) Stuurman, F. E.; Al, E. *Clinical Pharmacology of Novel Anticancer Drug Formulations*; 2013.
- (67) Kalepu, S.; Nekkanti, V. Insoluble Drug Delivery Strategies: Review of Recent Advances and Business Prospects. *Acta Pharmaceutica Sinica B.* 2015, pp 442–453.
- (68) Sawicki, E.; Schellens, J. H. M.; Beijnen, J. H.; Nuijen, B. Inventory of Oral Anticancer Agents: Pharmaceutical Formulation Aspects with Focus on the Solid Dispersion Technique. *Cancer Treat. Rev.* **2016**, *50*, 247–263.
- (69) Narvekar, M.; Xue, H. Y.; Eoh, J. Y.; Wong, H. L. Nanocarrier for Poorly Water-Soluble Anticancer Drugs—Barriers of Translation and Solutions. *AAPS PharmSciTech* **2014**, *15* (4), 822–833.
- (70) López-Castillo, C.; Rodríguez-Fernández, C.; Córdoba, M.; Torrado, J. J. Permeability Characteristics of a New Antifungal Topical Amphotericin B Formulation with γ -Cyclodextrins. *Molecules* **2018**.
- (71) Goyal, U.; Gupta, A.; Rana, A. C.; Aggarwal, G. Self Microemulsifying Drug Delivery System: A Method for Enhancement of Bioavailability. *International Journal of Pharmaceutical Sciences.* 2012, pp 66–79.
- (72) Lo, J. T.; Lee, T. M.; Chen, B. H. Nonionic Microemulsions as Solubilizers of Hydrophobic Drugs: Solubilization of Paclitaxel. *Materials (Basel)*. **2016**, *9* (9).
- (73) H, Z. Addressing Solubility through Nano Based Drug Delivery Systems. *J. Nanomed. Nanotechnol.* **2016**, *7* (3), 1–7.
- (74) Savjani, K. T.; Gajjar, A. K.; Savjani, J. K. Drug Solubility: Importance and Enhancement Techniques. *ISRN Pharm.* **2012**, *2012* (February 2015), 1–10.

CHAPTER-2

IONIC-LIQUID-BASED PACLITAXEL PREPARATION: A NEW POTENTIAL FORMULATION FOR CANCER TREATMENT

2.1 Abstract

Paclitaxel (PTX) injection (i.e. Taxol) has been used as an effective chemotherapeutic treatment for various cancers. However, the current Taxol formulation contains Cremophor EL, which causes hypersensitivity reactions during intravenous administration and precipitation by aqueous dilution. This article reports the preliminary results on the ionic liquid (IL)-based PTX formulations developed to address the aforementioned issues. The formulations were composed of PTX/cholinium amino acid ILs/ethanol/tween-80/water. A significant enhancement in the solubility of PTX was observed with considerable correlation with the density and viscosity of the ILs, and with the side chain of the amino acids used as anions in the ILs. Moreover, the formulations were stable for up to 3 months. The driving force for the stability of the formulation was hypothesized to be the involvement of different types of interactions between the IL and PTX. *In vitro* cytotoxicity and anti-tumor activity of the IL-based formulations were evaluated on HeLa cells. The IL vehicles without PTX were found to be less cytotoxic than Taxol, while both the IL-based PTX formulation and Taxol exhibited similar antitumor activity. Finally, *in vitro* hypersensitivity reactions were evaluated on THP-1 cells and found to be significantly lower with the IL-based formulation than Taxol. This study demonstrated that specially designed ILs could provide a potentially safer alternative to Cremophor EL as an effective PTX formulation for cancer treatment giving fewer hypersensitivity reactions.



Graphical Presentation of IL-mediated PTX Formulation.

2.2 Introduction

For effective therapeutic management of diseases, the discovery and development of new drugs alone are not adequate. Modification and improvement of pharmaceutical formulations of existing water soluble/insoluble drugs have gained great attention. Paclitaxel (PTX) was originally isolated from the bark of the Western Yew (*Taxus brevifolia*) in the late 1960s by Monroe Wall and his collaborators,¹ and it has been considered as the most widely used chemotherapeutic agent for the treatment of various types of cancers² (breast, ovarian and lung) owing to its unique mechanism of action.^{2,3} However, the very poor water solubility (<4 µg/mL) of PTX has compromised its ubiquitous applications as an anticancer drug,^{1,4} necessitating the development of the parenteral formulation Taxol.^{5,6} Taxol consists of Cremophor EL (CrEL, ethoxylated castor oil) and dehydrated ethanol (EtOH) of equal volumes.⁵ CrEL acts as a solubilizing agent,⁶ and it is not an inert substance, which can induce major hypersensitivity reactions and various undesirable side effects.^{5,6} Overcoming the side effects of CrEL or replacing it in the Taxol formulation has proved challenging. Attempted strategies to address the above problems include PTX-bound albumin⁷ or gold⁸ nanoparticles, microemulsion of PTX encapsulated by polyethylene glycol-poly lactide/D- α -tocopheryl polyethylene glycol succinate⁹ or conjugated with either docosahexaenoic acid,¹⁰ poly-L-glutamic acid¹¹ or angiopep-2 (brain peptide vector),¹² immunoliposomes composed by PEGylation¹³ or tween-80/HSPC/cholesterol,¹⁴ and micellar formation of block copolymers.¹⁵

For the last two decades, ionic liquids (ILs) have been designed as “green” solvents and have been used extensively in various applications including drug delivery and drug formulations.¹⁶ ILs have unusual physicochemical properties including excellent thermal and chemical stability, negligible vapor pressure, low flammability, non-volatility, high ionic conductivity, recyclability and multiple solvation abilities with organic and inorganic compounds. These extraordinary properties have rendered ILs very useful in biomedical applications (bio-catalysis,¹⁷ separations,¹⁸ energy storage and conversion,¹⁹ etc.) as well as in pharmaceutical applications¹⁶ as a potential component of drug formulations.²⁰ Examples of such formulations include the formation of active pharmaceutical ingredients (API) in an IL form (API-IL)²¹ and IL-containing solids in oil microemulsions²²/nanodispersions²³ for transdermal/topical drug delivery. It is well reported that ILs act as an excellent solubilizing agent for drug molecules that are sparingly soluble/practically insoluble in water.^{24–26} In fact, the major role of ILs is to solubilize and stabilize the drug molecule in drug delivery system. In case of PTX, an IL(TOMAC)-based CNT-Taxol-embedded PCL microspheres have been

reported by Kim et al. where the trioctylmethylammonium IL was removed post-processing to develop a sustained drug delivery system.²⁷ Here, we report a new PTX formulation with a mixture of biocompatible IL, tween-80 (polyoxyethylene 20 sorbitan monooleate; T-80), EtOH and H₂O as a delivery system in a specific ratio, where ILs enhance the solubility and stability of PTX in the formulation instead of CrEL.

2.3 Experimental

2.3.1 Materials and Methods

2.3.2 Materials

2.3.3 Chemicals

The anticancer drug paclitaxel was procured from Tokyo Chemical Industry Co., Ltd with purity of 99.9%. Choline chloride, glycine, L-alanine, L-phenylalanine, L-serine, L-proline, L-leucine, L-Isoleucine and EtOH (analytical grade) were purchased from Chameleon Guaranteed Reagent, Japan with high level of purity commercially available. Silver oxide, methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd. Tween-80 (T-80) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and Cremophor EL (polyethoxylated castor oil) were obtained from Fluka Biochemika, Germany. Ultra-pure water from Milli-Q system was used throughout the experiment. All chemicals for the entire experiment were used without further purification.

2.3.4 Cells, Medium and Reagents

HeLa cells: The human cervical cancer cells (HeLa cells) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were propagated as adherent monolayer in MEM medium (Gibco, Life Technologies Corp., USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies Corp., USA) and 0.1% of antibiotic-antimycotic (Gibco, Life Technologies Corp., USA).

THP-1 cells: The human leukemia cells (THP-1 cells) were procured from NIHS (JCRB) Cell Bank, Japan. Cells were cultured in RPMI-1640 medium (Nacalai Tesque Inc., Kyoto, Japan) with supplements of 10% heat-inactivated FBS, 0.05 mM 2-mercaptoethanol (Wako Pure Chemical Industries Ltd., Japan) and 1% of antibiotic-antimycotic.

Medium and Reagents: All other reagents for cells experiment were procured from miscellaneous sources: D-Phosphate Buffer Saline [D-PBS (-)] and OptiMEM from Gibco, Life Technologies Corp., USA; cell counting kit-8 (WST-kit) from Dojindo Co., Japan; 0.25% trypsin/1 mM EDTA solution from Nacalai Tesque, Inc., Japan; 1-chloro-2,4-dinitrobenzene from Sigma-Aldrich, UK, CAS-97-00-7 (Purity $\geq 99\%$); Nickel (II) Sulfate Hexahydrate from Sigma-Aldrich, USA, CAS-10101-97-0 (Purity $\geq 99.99\%$); L-(+)-lactic acid from Sigma-Aldrich, Belgium, CAS-79-33-4 (Purity $\geq 98\%$), albumin (bovine) from Nacalai Tesque Inc., Kyoto, Japan; propidium iodide (1000 $\mu\text{g/mL}$) from Djindo Co., Japan; anti-mouse CD16/32 (Fc receptor blocking antibody, clone: 93, isotype: rat IgG2a, $\square$), PE anti-human CD86 (clone: IT2.2, isotype: mouse IgG2b, $\square$) and PE anti-human CD54 (clone: HA58, isotype: mouse IgG2b, $\square$) from BioLegend, Inc., San Diego, CA, USA.

2.3.5 Synthesis of Choline Amino Acid Ionic Liquids ([Ch][AA] ILs)

Choline Amino Acid ([Ch][AA]) ILs were synthesized via a two-step metathesis reaction, with slight modification of a previously published procedure.²⁸ Briefly, in the first step, choline hydroxide ([Ch]OH) aqueous solution was synthesized from the metathesis of choline chloride ([Ch]Cl) (0.05 mol) with silver oxide (Ag_2O) (0.025 mol) using water as solvent with excess Ag_2O in the reaction medium. Then, excess Ag_2O and AgCl were removed from the medium by centrifugation (3500 rpm, 15 min) and filtration. In the second step, the filtrate containing [Ch]OH was neutralized with an equimolar aqueous solution of amino acid (AA) by stirring at room temperature for 24 h with excess AAs [Fig.2-S1]. The solvent was evaporated using rotary evaporator (EYELA, NVC-2200) at 40 °C for 3 h. After that, a mixture of acetonitrile/methanol (9:1, v/v) was added and stirred vigorously to obtain precipitation of unreacted AAs, which were then filtered off. The filtrated solution was evaporated again to remove the remaining solvents using rotary evaporator at 40 °C. Finally, the ILs were freeze-dried for 24 h to evaporate the residual solvents completely.

All synthesized ILs were characterized by ^1H NMR (JEOL Delta, ECZS NMR spectrometer, 400 MHz) spectroscopy and elemental analysis. The data obtained were in good agreement with literature values.^{28,29} Water contents of all ILs were determined by Karl Fischer (KF) titration and were found to be less than 0.5% (wt/wt). All data strongly supported and confirmed the successful syntheses of the seven [Ch][AA] ILs: choline glycine ([Ch][Gly]), choline alanine ([Ch][Ala]), choline phenylalanine ([Ch][Phe]), choline proline ([Ch][Pro]),

choline serine ([Ch][Ser]), choline leucine ([Ch][Leu]) and choline isoleucine ([Ch][Ile]). All ILs were liquids at room temperature and referred to as room temperature ILs (RTILs).

2.3.6 Physical Properties

Density & Viscosity: The instruments used for determination of physical properties were calibrated using Millipore-quality water before taking the reading as instructed by the supplier. An Anton Paar Microviscometer (Lovis, 2000M/ME, Austria) and Anton Paar Density Meter (DMA 35N, Austria) were used to measure the viscosity and density of ILs at a temperature of $25\text{ }^{\circ}\text{C} \pm 0.01\text{ }^{\circ}\text{C}$. The viscometer was calibrated using water as a solvent, and about 1.3 mL of each IL was added to the sample tube for measurement. The thermal equilibration time was set at about 15 min. The densimeter was calibrated under dry air, the bidistilled water was degassed and the density of each IL was measured at ambient pressure and temperature. The precision of the densimeter was recorded as $5 \times 10^{-4}\text{ g.cm}^{-3}$.

2.3.7 Solubility of Paclitaxel (PTX) in [Ch][AA] ILs

The solubility study was performed by adding an excessive amount (12.0–15.0 mg) of PTX to a glass vial containing 0.5 g of each IL separately, stirring at ambient temperature for 12 h. The undissolved PTX remained as precipitates, which were removed by high centrifugation (15000 rpm, 30 min) and filtration of the upper layer through a $0.45\text{ }\mu\text{m}$ filter (Millipore Millex-LG, PTFE, Fisher Scientific, USA). The clear filtrated solutions were analyzed using a UV spectrophotometer (JASCO V-670, USA) at 239 nm to estimate the amount of PTX dissolved in ILs. Following a literature validation method,³⁰ ILs were diluted in 1:1 methanol and phosphate buffer (pH 6.8) and used as the reference standards.

2.3.8 [Ch][AA] IL Based PTX Formulation

PTX loaded ILs were treated with saline (0.9% NaCl) or PBS (pH 6.8) or water and found to give precipitations of PTX. A composition of IL/EtOH or IL/T-80 or EtOH/T-80 was then used in the preparation of a PTX aqueous solution, and similar precipitations were observed. Finally, a clear, particle-free PTX aqueous solution was achieved with EtOH as a solvent and both IL and T-80 as co-solvents. Briefly, 0.5 mg of PTX was dissolved in 100 μL of EtOH. To this solution, 50 μL of IL and 50 μL of T-80 were added and the mixture was mixed gently to

obtain a homogeneous solution which was further diluted in 800 μ L of either saline (0.9% NaCl) or PBS (pH 6.8) or water to obtain a clear, particle-free PTX solution.

2.3.9 *In vitro* Evaluation of Cytotoxicity of IL-Based Formulations in Human Cervical Cancer Cells (HeLa cells)

The cytotoxicity of all IL-based formulations were evaluated using HeLa cells cultured in MEM with 10% FBS and 0.1% AA solution at 37 °C in a humidified atmosphere of 5% CO₂. During the experiment, the medium was changed every 2 days and the cells were sub-cultured at the confluence of approximately 80% or above. Briefly, the cells were washed with D-PBS(-) two times and trypsinized by 1.0 mL of 0.25% trypsin/1 mM EDTA solution and incubated for 3 min at 37 °C. The cells were then rinsed with D-PBS(-) two times and resuspended at a concentration of approximately 2×10^3 cells/ mL. To check the cytotoxic effect of the formulations, a colorimetric assay of WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt] was used for the HeLa cell viability test. After trypsinization, the cells were counted and plated in a 96-well plate, where each well contained 5000 cells in 100 μ L of the medium. 5% (w/v) of stock solutions of different formulations (Table 2-S1) were initially prepared with OptiMEM medium. In the case of CrEL, a second dilution was also performed in a 20:80 ratio with PBS (pH 7.4) before diluting in OptiMEM.

The samples were prepared at concentrations ranging from 0.02% to 5.0% (w/v) and after 24 h, were added to the wells up to a final volume of 100 μ L. The incubation was performed in triplicates for each sample including the control. After 24-hour incubation, the cells were washed twice with D-PBS(-) and again diluted by 100 μ L of (11 times diluted by OptiMEM) WST-1 and incubated for 3 h at 37 °C. Finally, the optical density was measured at 450 nm in a microplate reader (iMark, Bio-Rad, USA). Cell viability was calculated as the percentage of exposed cells that remained viable over controls. The data were collected from at least three independent experiments and used to calculate the mean value.

2.3.10 Size Distribution by DLS Measurement

The presence of PTX particles and their size distributions in all formulations (similar to Table 2-S2, except containing 0.5 mg/mL of PTX) were evaluated by a Laser Diffraction Particle Size Analyzer (Nano Series, ZS MALVERN, U.K.), a well-computerized inspection system at nano-scale. All samples were further diluted (1:2) in a dispersing medium (deionized

H₂O) before measurement. The experiment was carried out at 25 °C ± 0.1 °C. The wavelength of the light source was kept at 633 nm with a scattering angle of 90°.

2.3.11 *In vitro* Precipitation Test

A simple *in vitro* precipitation detection method using 96-well microplates and a SpectraMax Plus microtiter plate reader (Molecular Devices, LLC, Sunnyvale, USA) was used following a previously published procedure³¹ with slight modification. Briefly, a small volume (100 µL) of each formulation (Table 2-S3) was added to a 96-well microtiter plate. Equal volumes (100 µL) of PBS (pH 7.4) were added giving progressive dilution of the formulations. The optical density (OD at 500) of the wells was measured in the same order as the dilution process, using a SpectraMax Plus microtiter plate reader equipped with the SoftMax Pro software. OD was measured at 0.0 min, 15.0 min, 30.0 min, 60.0 min, 3.0 h, 6.0 h, 12.0 h and 24.0 h (reading point = 7). The experiment was carried out at 25 °C ± 0.1 °C. The stability of the formulations was monitored up to 3 months at 7 days, 15 days, 30 days, 45 days, 60 days and 90 days.

2.3.12 *In vitro* Evaluation of Anti-Tumor Activity of [Ch][Gly] IL-Based Formulations in HeLa Cells

The anti-tumor activity of IL-based formulation was evaluated in HeLa cells. Briefly, five different formulations were prepared as described in Table 2-S4, where the first two consisted of 0.5 mg/mL of PTX and the rest were vehicles only and referred to as placebo. The concentrations of all the samples used in the study ranged from 0.02 to 20.0 % (w/v) of formulation, which corresponds to 0.005–20.0 µg/mL of PTX. The WST-1 assay, as described for *in vitro* evaluation of cytotoxicity of IL-based formulations, was conducted to measure the anti-tumor efficacy of the formulations.

2.3.13 *In vitro* Evaluation of Hypersensitivity of [Ch][Gly] IL-Based Formulations by Human Cell Line Activation Test (h-CLAT)

The h-CLAT experiment was conducted according to a literature procedure with slight modification.^{32,33}

2.3.14 Cytotoxicity Test and Determination of CV75

THP-1 cells were seeded in 96-well plates (1.6× 10⁵ cells/0.1 mL/well) with eight different concentrations of placebo formulations of CrEL(–)PTX and IL/T-80(–)PTX, with 2-fold serial dilution starting from 100 µL/mL for 24 h. The negative control and diluent used in the CV75 experiment was the cell's culture medium. Formulation-treated cells were washed two times

with D-PBS(-) solution containing 0.1% (w/v) bovine serum albumin (FACS buffer). After staining with propidium iodide (PI, 0.625 µg/mL) solutions, the cells were analyzed using a flow cytometer (Sony EC800 Cell Analyzer, Biotechnology Co., Ltd., Japan) with the acquisition channel FL-2 where PI negative (PI-) cells were defined as living cells. Cell viability was calculated using the cytometer analysis program. The CV75 value (a concentration showing 75% of THP-1 cell survival) was determined using the fitting graph equation $Y = a + (b - a) / \{1 + (c/X)^d\}$, where Y represents the relative cell viability (% of cell survival), X represents the concentration of formulation, and a, b, c, and d are constants for the fitting graph estimated statistically.

2.3.15 Measurement of Cell Surface Markers: Expression of CD86 and CD54

The CV75 value was used to determine the concentration (% w/v) of placebo formulations in the measurement of CD86/CD54 expression. THP-1 cells were exposed to negative control (lactic acid), IL/T-80(-)PTX, CrEL(-)PTX and positive controls [2,4-dinitrochlorobenzene (DNCB) & nickel sulfate] at eight concentrations based on the CV75 (Table 2-S5): $1.2 \times CV75$, $1 \times CV75$, $1/1.2 \times CV75$, $1/1.2^2 \times CV75$, $1/1.2^3 \times CV75$, $1/1.2^4 \times CV75$, $1/1.2^5 \times CV75$ and $1/1.2^6 \times CV75$. The cells were washed twice with FACS buffer 24 h after treatment. The cells were then incubated with 200 µL of Fc receptor blocking solution (0.01% of globulin in FACS buffer) for 15 min at 4 °C. The cells were stained with 50 µL of PE-labelled anti-CD86 and anti-CD54 antibodies for 30 min at 4 °C separately and diluted in a ratio of 3:25 (v/v) for CD86 and 3:50 (v/v) for CD54 with FACS buffer. After incubation, the cells were resuspended in 200 µL of FACS buffer after washing three times and 10 µL of the PI solution was added (final concentration of PI was 0.625 µg/mL). Cell viability was calculated from the PI- negative area of the FL-2 channel by using the default cytometer analysis program and the protein expression was estimated by the geometric mean of the PE intensity. The relative fluorescent intensity (RFI) was calculated using the following formula:

$$RFI(\%) = 100 \times (MFI \text{ of chemical-treated cells} - MFI \text{ of chemical-treated isotype}) / (MFI \text{ of vehicle control cells} - MFI \text{ of vehicle isotype control cells})$$

where RFI is an indicator of relative expression of CD86 or CD54 and MFI refers to the geometric mean fluorescent intensity obtained from flow cytometry.

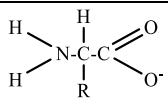
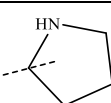
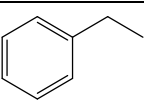
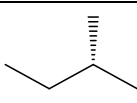
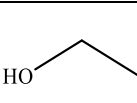
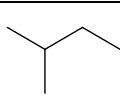
The estimated concentration required to obtain a %RFI of 150 and 200 for CD86 and CD54 (EC150 and EC200) respectively was calculated for both the lowest dose above the criterion for a positive response and the highest dose below the criterion.

2.4 Results and Discussion

First, we investigated the solubility of PTX in seven biocompatible ILs consisting of choline (Ch) as the cation and amino acid (AA) Gly, Ala, Ser, Leu, Ile, Pro or Phe as the anion.³⁴ The biocompatibility or toxicity of an IL mostly depends on cation with minor anionic effect. It has been reported that ILs containing ammonium and alicyclic cations (morpholinium, piperidinium, and pyrrolidinium) are more compatible compared to imidazolium and pyridinium cations. Furthermore, the incorporation of polar hydroxyl/ether/nitrile functional group into the alkyl chain of cation significantly enhance the biocompatibility. In our study, both the cation, cholinium, which is derived from choline, an essential micronutrient that can be completely metabolized due to having a polar hydroxyl group, and the anion AAs, most abundant classes of organic molecules in nature, are considered as nontoxic, biodegradable and biocompatible.^{34,35}

Upon investigation, we observed significantly enhanced solubility of PTX in ILs, with the highest solubility (22.34 mg/mL) in [Ch][Gly] IL and the lowest (6.61 mg/mL) in [Ch][Leu] IL, compared with $<4 \mu\text{g/mL}$ ⁴ in water (Fig.2-1A & 2-1B). As shown in Table 2-1, the solubility of PTX is highly dependent on the viscosity and density of the IL, as well as the size of the anions. It has been reported that the dissolution ability of ILs is significantly associated with density³⁶ and viscosity,³⁷ while the presence of bulky/aromatic side chains in the anion reduces dissolution.³⁷ ILs are believed to mediate dissolution of hydrophobic molecules through the anions which directly bind to the molecules via hydrogen bonding, van der Waals force or π - π interactions.^{38,39} Small anions containing localized charges have reportedly higher dissolution effects owing to the strong proton accepting ability.³⁶ While the cation interacts with the apolar part of the drug molecule to form hydrophobic interactions, the contribution of the cation to dissolution is relatively small.⁴⁰ Here, glycine is the simplest and smallest AA having high surface electron density than other AAs. PTX is easily and strongly partaken in [Ch][AA]-PTX complex formation to enhance PTX dissolution.

Table 2-1. Solubility of Paclitaxel (PTX) in ILs at room temperature and the effect of viscosity, density and anionic domain on IL-mediated dissolution^a

IL: [Ch][AA]	[Gly]	[Ala]	[Pro] ^b	[Phe]	[Ile]	[Ser]	[Leu]
	R= -H	-CH ₃					
Solubility of PTX (mg/ml)	22.34±0.86	18.52±0.88	16.16±0.26	14.15±0.026	9.39±0.21	7.32±0.15	6.61±0.17
Density (ρ,g.cm⁻³)	1.1526±0.004	1.1343±0.008	1.1379±0.003	1.1411±0.001	1.0705±0.034	1.1263±0.005	1.0994±0.024
Viscosity (ρ,g.cm⁻³)	1.28±0.06	0.83±0.10	9.09±0.64	49.61±5.05	11.11±0.46	12.37±0.61	8.24±0.27

^a Data are shown as mean ± SD (n=3).^b Proline does not uphold the ordinary AA structure

Preparation of PTX loaded [Ch][AA] IL was initially attempted by adding aqueous solvents such as water, PBS (pH 7.4) or saline (0.9% NaCl). However, clearly visible particles/precipitations were observed just after the addition of aqueous solvents because of the release of PTX from the [Ch][AA]-PTX complex. It should be noted that all seven ILs used in this study were hydrophilic in nature, which released the drug molecule from the IL-PTX complex in the presence of hydrophilic solvents.

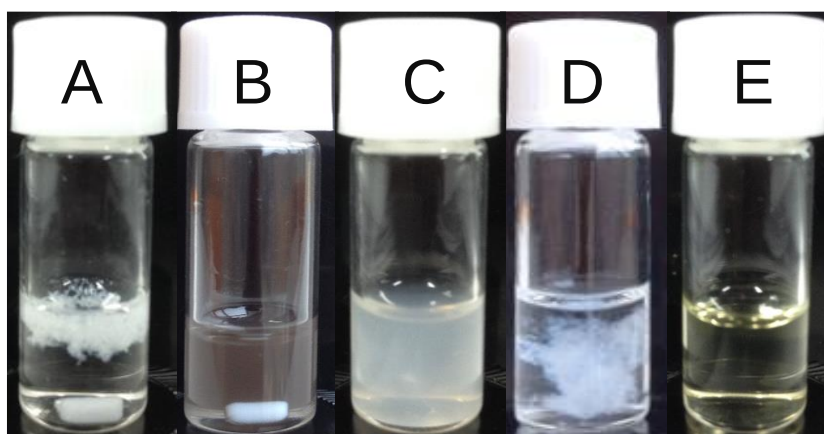


Figure 2-2. PTX (0.5 mg/mL) in different solvent systems (A) H₂O; (B) IL; (C) T-80/EtOH/H₂O; (D) IL/EtOH/H₂O; (E) IL/T-80/EtOH/H₂O

The use of multiple co-solvents in pharmaceutical formulations, especially for injection of a hydrophobic drug, is very common and widely accepted.³¹ The basic requirements for this are as follows: a) co-solvents should be freely miscible in an aqueous system; b) the hydrophobic drug must be soluble in the co-solvents, and c) the co-solvents should be biocompatible and approved for pharmaceutical applications. Therefore, EtOH and tween-80,

which fulfill these basic requirements, were selected as additional co-solvents for this study. Because PTX is highly soluble in EtOH, in a later experiment PTX was initially dissolved in EtOH and the dissolution effect of [Ch][AA] IL or T-80 was then evaluated in the formulation. The aggregates/precipitates of PTX were observed with any combination of EtOH, IL, and T-80, but not when all of them were used in the solvent system (Fig.2-1).

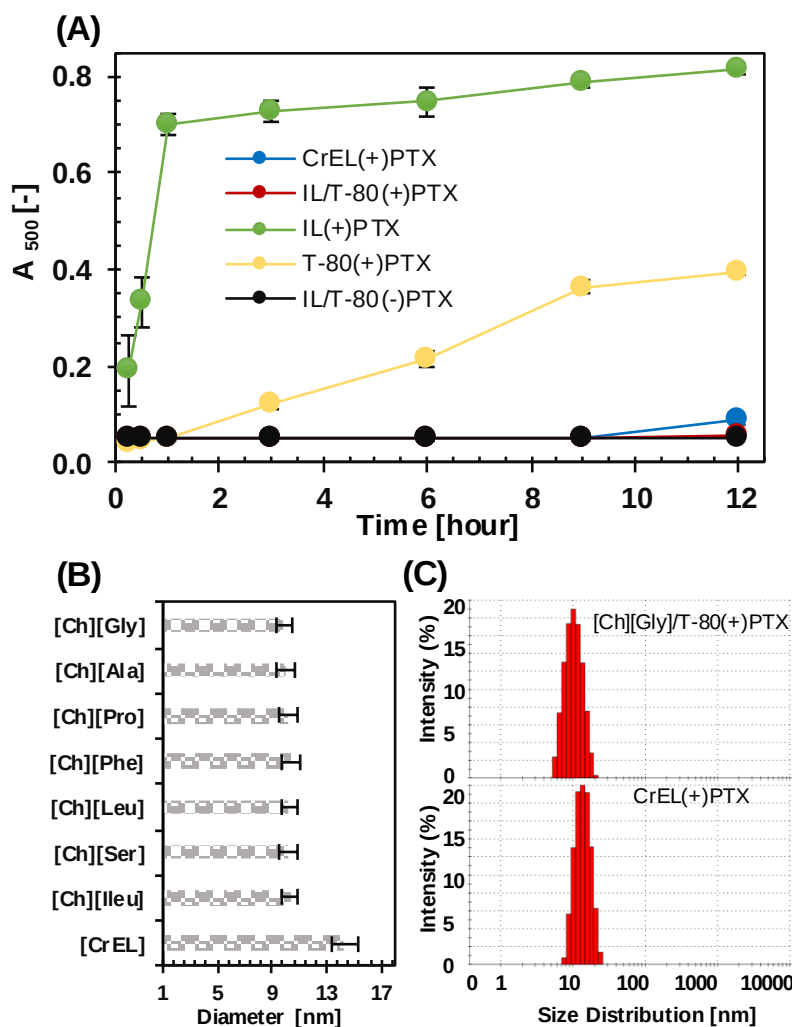


Figure 2-3. (A) *In vitro* precipitation study of PTX aggregation in different solvent systems. Absorbance (at 500 nm) of different formulations (Table 2-S3) has been recorded up to 12 h. (B) Typical particle sizes (diameter, nm) of PTX formulations (Table 2-S2). (C) Size distribution profile of IL-based formulation and CrEL.

To confirm the nonappearance of aggregates²⁶ in the IL-based formulation, we performed an *in vitro* precipitation test of different formulations, (Table 2-S3) where the escalation of absorbance due to the amendment of opacity of the solution by precipitations of PTX was scrutinized at 500 nm.³¹ The absorbance remained unaffected up to 12 hours in the IL-based formulation (any of the seven ILs) as well as in CrEL but not for a formulation that lacked

either IL or T-80 (Fig.2-1 & 2-2A). Furthermore, the particles were found to be nano-sized for all IL-based formulations and CrEL via dynamic light scattering (DLS) measurement (Fig.2-2B & 2-2C). The formulations (Table 2-S2) were monitored and found to be stable for up to 3 months. The driving force for the stabilization of the formulations was hypothesized to be the involvement of the different types of solvents/co-solvents and their interactions with the PTX molecule. As PTX forms multiple strong intermolecular hydrophobic interactions (hydrogen bonding, van der Waals force or π - π stacking) with the co-solvents, a hydrophobic pocket in the PTX molecule could be created where PTX would be surrounded by these co-solvents that eventually inhibit PTX self-aggregation in aqueous solutions. The high surface energy of the nano-sized particles is responsible for their aggregation, which can be inhibited by the addition of stabilizers and other excipients in aqueous solutions.⁴¹ We hypothesized that [Ch][AA] ILs together with T-80 and EtOH act as stabilizers providing a steric and ionic barrier to PTX self-aggregation.

The most important criterion of a vehicle for any drug delivery system is safety, thus we evaluated the cytotoxicity of the placebo formulations in WST assays against HeLa cells. Cell viability was significantly higher for IL-based formulations compared with CrEL even in 20:80 dilution (Table 2-S1) (Fig.2-3). In the proposed formulation where CrEL was replaced by T-80 and IL as an alternative vehicle system for PTX delivery, the addition of IL to the vehicle had no impact on cancer cells; furthermore, it lowered the toxicity on HeLa cells. It was reported that changes in the anionic component from a small methyl group to a longer ethyl chain significantly increased the EC50 value of the latter IL,³⁶ and branching side chains were associated with reduced toxicity.³⁶ The elongation of the linear chain supposedly enhanced the lipophilic nature of ILs resulting in increased toxicity whereas branching chains were considered to reduce lipophilicity.³⁶ Glycine with no side chain showed the lowest cytotoxicity whereas AAs with aromatic/cyclic side chains showed considerably higher toxicity. Incorporation of the aromatic system or cyclic ring greatly influences the cellular binding of IL on the membrane causing subsequent cell damage.⁴² The cytotoxicity in descending order was as follows: [CrEL]> [Ch][Phe]> [Ch][Pro]> [Ch][Leu]> [Ch][Ser]> [Ch][Ile]> [Ch][Ala]> [Ch][Gly], which was in good agreement with the literature.^{42,34}

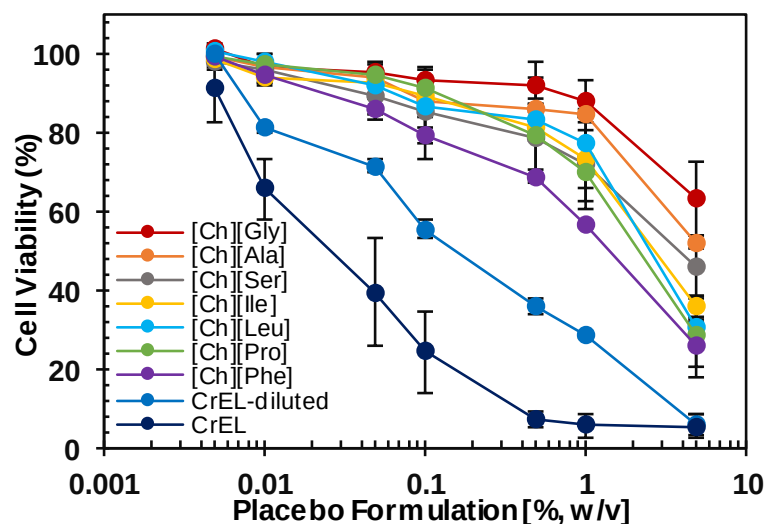


Figure 2-4. Viability of HeLa cells treated with various concentrations of IL-based placebo formulations or CrEL, in the absence of PTX.

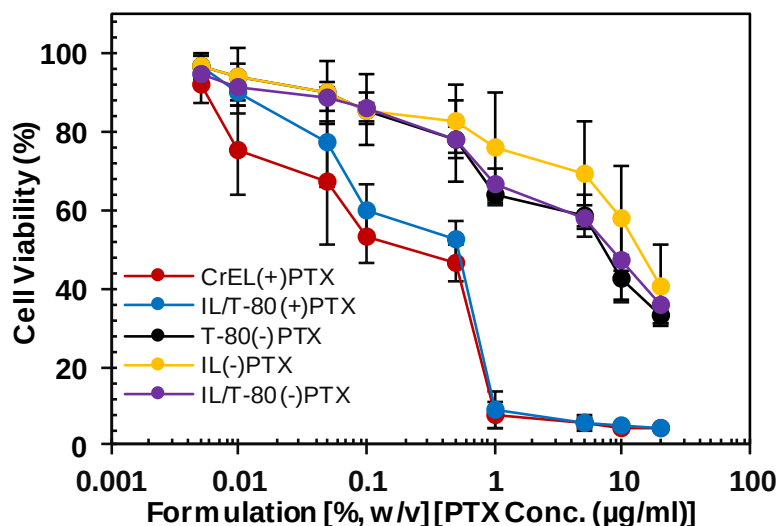


Figure 2-5. Viability of HeLa cells treated with various concentrations of formulations with or without PTX.

The antitumor activity of the formulations with or without PTX (Table 2-S4) was evaluated in HeLa cells. Both PTX-containing formulations showed a similar cytotoxicity profile in a dose-dependent manner. The cytotoxic effects were almost identical when PTX concentration was above 1 $\mu\text{g/mL}$ (Fig.2-4). When PTX concentration was below 1 $\mu\text{g/mL}$, slightly higher cytotoxicity was observed with CrEL(+)-PTX than IL/T-80(+)-PTX. The significantly lower cytotoxicity of PTX free formulations compared with CrEL(+)-PTX and IL/T-80(+)-PTX during

the test period indicated that the antitumor activity of the formulations was due to the presence of PTX.

Finally, the human cell line activation test (h-CLAT) was carried out to determine the expression of CD86 or CD54, the markers of hypersensitivity, in human leukemia cell lines (THP-1).⁴³⁻³⁹ In this experiment, two well-known sensitizers, 2,4-dinitrochlorobenzene (DNCB) and nickel sulfate (NiSO₄) were used as positive controls and the non-sensitizer lactic-acid was used as a negative control along with IL/T-80(-)PTX and CrEL(-)PTX. The expression of CD86 and/or CD54 on THP-1 cells was significantly increased when treated with sensitizers used as an indicator of hypersensitivity.^{33,44} A relative fluorescent intensity (RFI) of $\geq 200\%$ and 150% for CD86 and CD54 respectively was considered as a positive response.⁴⁵ The calculation of RFI was described in the supporting information. Briefly, eight different doses of each test samples (lactic-acid, IL/T-80(-)PTX, CrEL(-)PTX, DNCB and NiSO₄) based on dilutions from CV75 (concentration that showed 75% cell survival, Table 2-S5) were plotted on the X-axis against RFI to illustrate the expression of cell surface markers CD86 and CD54 (Fig.2-5A&2-5B). RFI was significantly higher when THP-1 cells were treated with either positive controls (DNCB or NiSO₄) or CrEL(-)PTX indicating that these are hypersensitive materials. The expression of CD86 and CD54 in these positive samples was found to be dose-dependent but was suppressed at high doses because of the relatively high cytotoxicity. No significant CD86 or CD54 expression was observed for the negative control (lactic acid) and IL/T-80(-)PTX. The RFI of CD54 for IL/T-80(-)PTX was found to increase slightly at $1.2 \times CV75$. The minor effect was reasonably due to the presence of T-80 in the formulation. The expression of both CD86 and CD54 with CrEL(-)PTX was significantly higher than that with IL/T-80(-)PTX, supporting higher hypersensitivity effects of CrEL(-)PTX than IL/T-80(-)PTX (Fig.2-5A &5B).

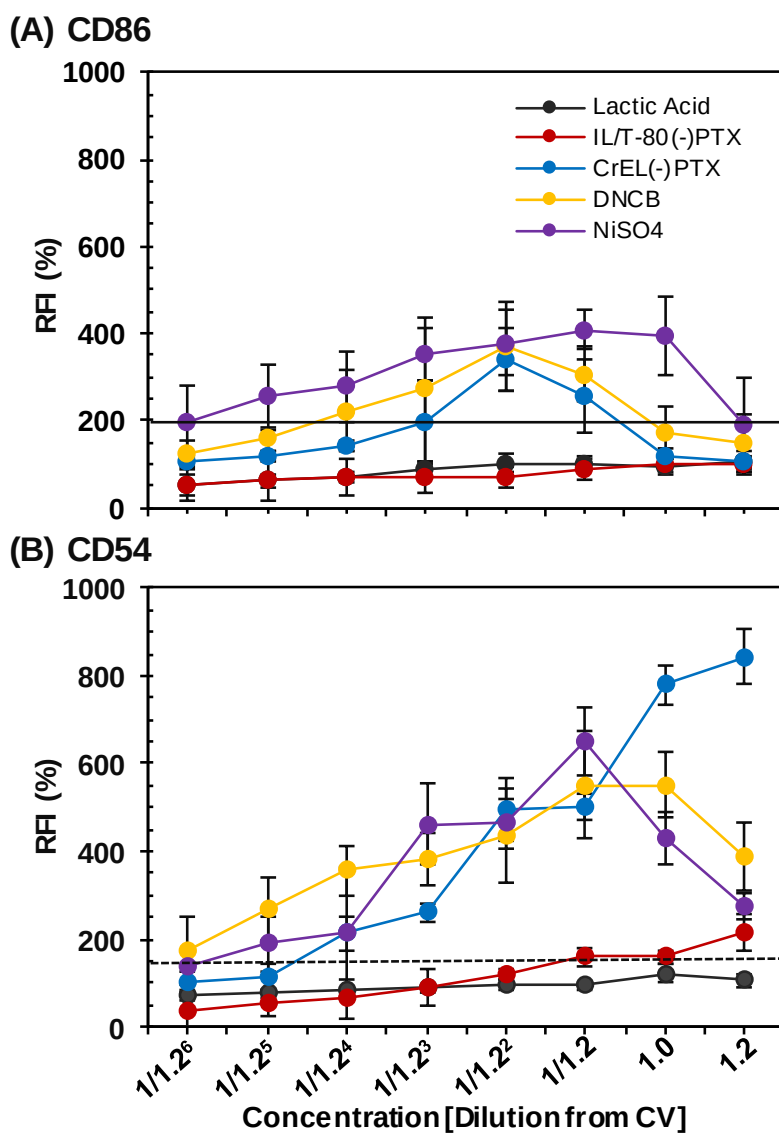


Figure 2-6. Expression of cell surface markers (A) CD86 and (B) CD54 (%RFI) on THP-1 cells treated with the test formulations (Table 2-S5) as an indication of hypersensitivity. RFI above 150% and 200% are considered positive for CD86 and CD54 respectively.

2.5 Conclusions

In summary, an IL-based, CrEL-free, PTX formulation was successfully developed as an effective alternative to the commercially available formulation. Compared with Taxol, this IL-based formulation had less toxicity but similar antitumor activity against HeLa cells. The hypersensitivity effect was also found to be significantly lower than Taxol. The preliminary investigations suggested that specially designed ILs could be an alternative vehicle (solvent/co-solvent systems) to conventional organic solvents for pharmaceutical formulations. Further investigation of the formation of intermolecular interactions between ILs and the drug molecule and the solvents/co-solvents is needed. More preclinical and clinical studies of the developed IL-based drug formulation are also necessary to fully realize the value of the formulation.

Supporting Data for Chapter-2

Tables

Table 2-S1. Compositions of different formulations without PTX for cytotoxicity study in HeLa cells.

Entry No.	Formulation Denotation	Compositions Ratio (% , w/v)				
		EtOH	IL	T-80	H ₂ O	CrEL
1	CrEL	50	-	-	-	50
2	CrEL-diluted	10	-	-	80	10
3	[Ch][Gly]	10	5	5	80	-
4	[Ch][Ala]	10	5	5	80	-
5	[Ch][Phe]	10	5	5	80	-
6	[Ch][Ser]	10	5	5	80	-
7	[Ch][Pro]	10	5	5	80	-
8	[Ch][Leu]	10	5	5	80	-
9	[Ch][Ile]	10	5	5	80	-

Table 2-S2. Compositions of different PTX formulations for DLS measurement.

Entry No.	Formulation Denotation	Compositions Ratio (% w/v)					PTX (mg/ml)
		EtOH	IL	T-80	H ₂ O	CrEL	
1	[CrEL]	50	-	-	-	50	0.5
2	[Ch][Gly]	10	5	5	80	-	0.5
3	[Ch][Ala]	10	5	5	80	-	0.5
4	[Ch][Phe]	10	5	5	80	-	0.5
5	[Ch][Ser]	10	5	5	80	-	0.5
6	[Ch][Pro]	10	5	5	80	-	0.5
7	[Ch][Leu]	10	5	5	80	-	0.5
8	[Ch][Ile]	10	5	5	80	-	0.5

Table 2-S3. Compositions of different formulations for *in vitro* precipitation study.

Entry No.	Formulation Denotation	Compositions Ratio (% w/v)					PTX (mg/ml)
		EtOH	IL	T-80	H ₂ O	CrEL	
1	CrEL(+)PTX	50	-	-	-	50	0.5
2	IL/T-80(+)PTX	10	5	5	80	-	0.5
3	IL(+)PTX	10	5	-	85	-	0.5
4	T-80(+)PTX	10	-	5	85	-	0.5
5	IL/T-80(-)PTX	10	5	5	80	-	0

Table 2-S4. Compositions of different formulations for the anti-tumor efficacy study in HeLa cells.

Entry No.	Formulation Denotation	Compositions Ratio (% , w/v)					PTX (mg/ml)
		EtOH	IL	T-80	H ₂ O	CrEL	
1	CrEL(+)PTX	50	-	-	-	50	0.5
2	IL/T-80(+)PTX	10	5	5	80	-	0.5
3	IL(-)PTX	10	5	-	85	-	0
4	T-80(-)PTX	10	-	5	85	-	0
5	IL/T-80(-)PTX	10	5	5	80	-	0

Table 2-S5. Dose setting (CV75) for the h-CLAT experiment.

Test Chemicals	CV 75 (µg/ml)	vehicle for h-CLAT	Source
Lactic acid	2800	Saline	Reference ³²
IL/T-80(-)PTX	13560	Saline	Experimental
CrEL(-)PTX	3580	Saline	Experimental
DNCB	6	DMSO	Reference ³²
NiSO ₄	150	Saline	Reference ³²

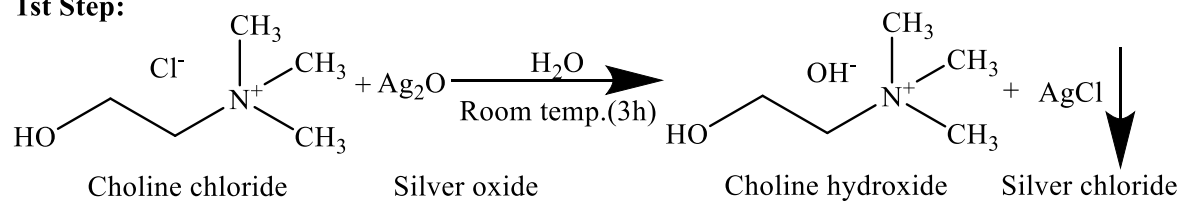
Table 2-S6. Size distribution, zeta potentials and conductivity of different formulations.

Formulation Denotation	Appearance	Z-average (d. nm) (PDI)	Z-potential (mV)	Conductivity (mS/cm)
CrEL(+)PTX	Clear	13.31±0.95 (0.029)	-0.80±0.14	0.20±0.04
IL/T-80(+)PTX	Clear	10.36±0.62 (0.046)	-2.21±0.31	9.83±0.27
T-80(+)PTX	Clear	2562±98 (0.530)	-1.97±0.24	0.05±0.04
IL(+)PTX	Not clear	ND	ND	ND

ND=Not Detected. PDI=Polydispersity Index

Figures

1st Step:



2nd Step:

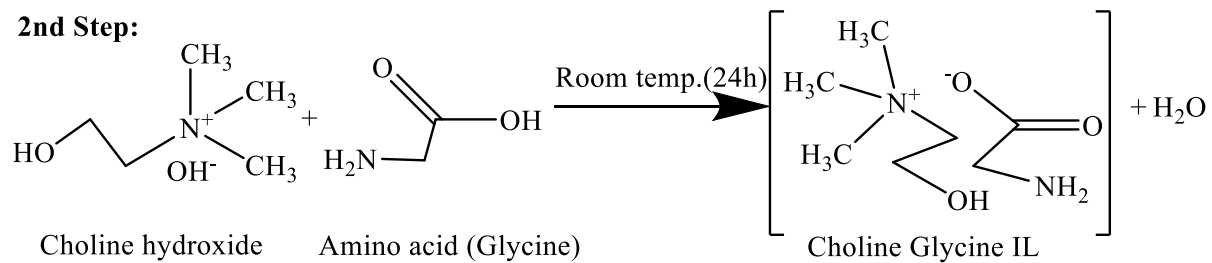


Figure 2-S1. Synthetic route for [Ch][AA] IL (Glycine as an example).

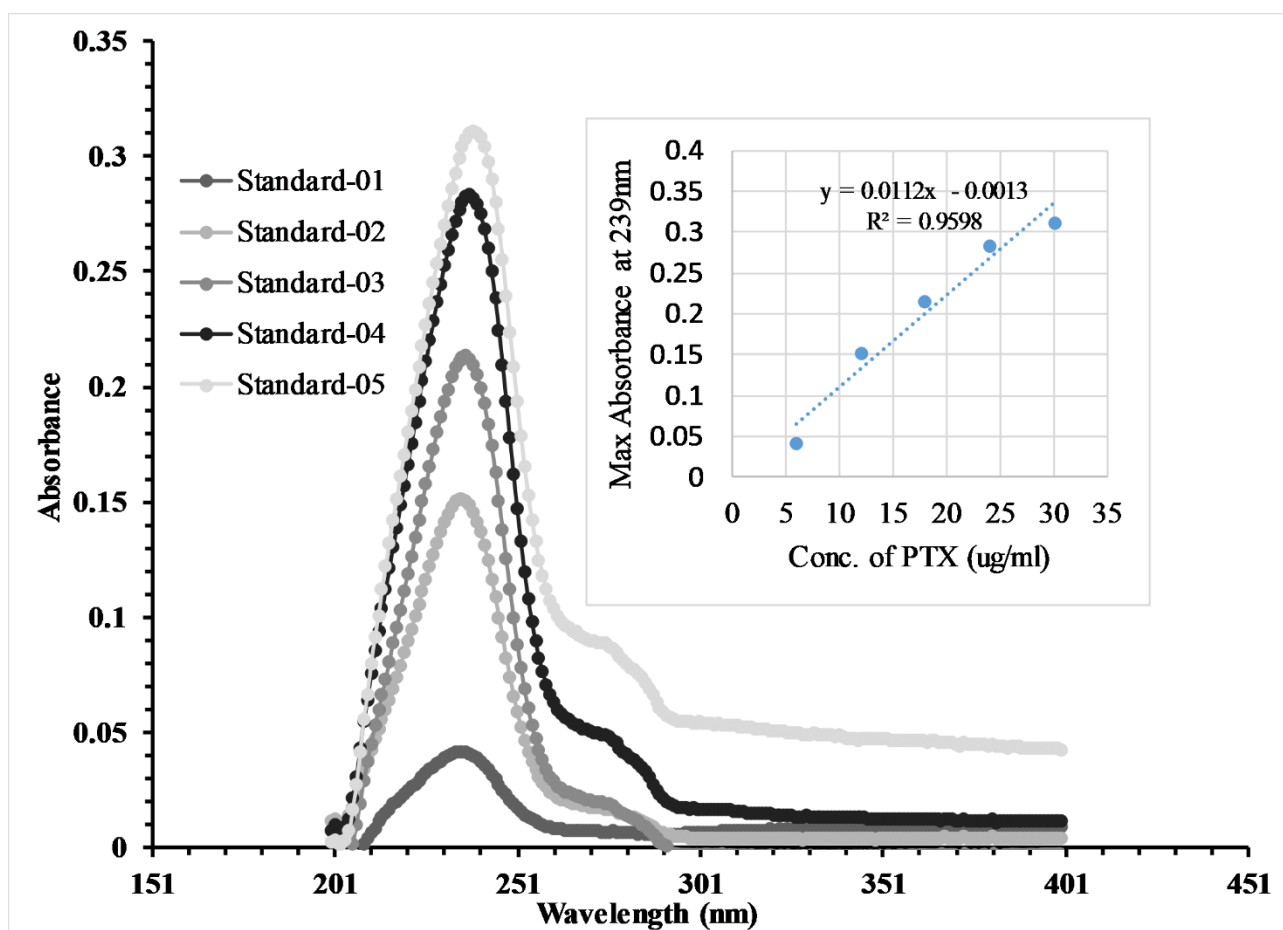


Figure 2-S2. Overlaid UV spectra of increasing concentrations (6–30 $\mu\text{g/mL}$) of standard PTX solutions; inset shows a plot of absorbance at 239 nm against concentrations of PTX in IL ($\mu\text{g/mL}$).

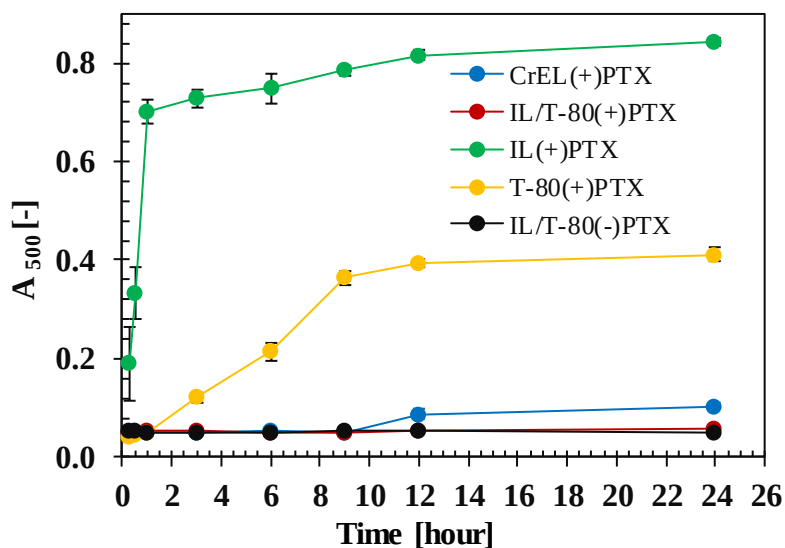


Figure 2-S3. *In vitro* precipitation study. Maximum absorbance (at 500 nm) of different formulations (Table 2-S3) at different time points up to 24 h.

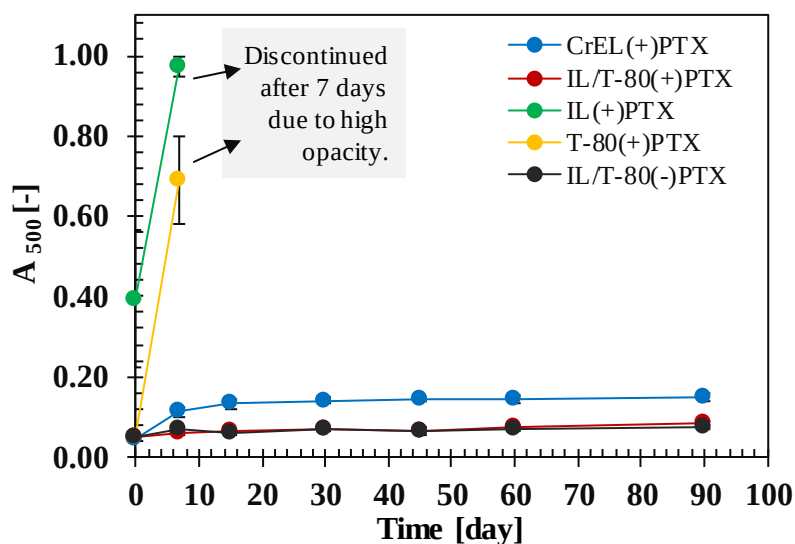


Figure 2-S4. *In vitro* stability study of different formulations (Table 2-S3) up to 3 months by in-vitro precipitation study.

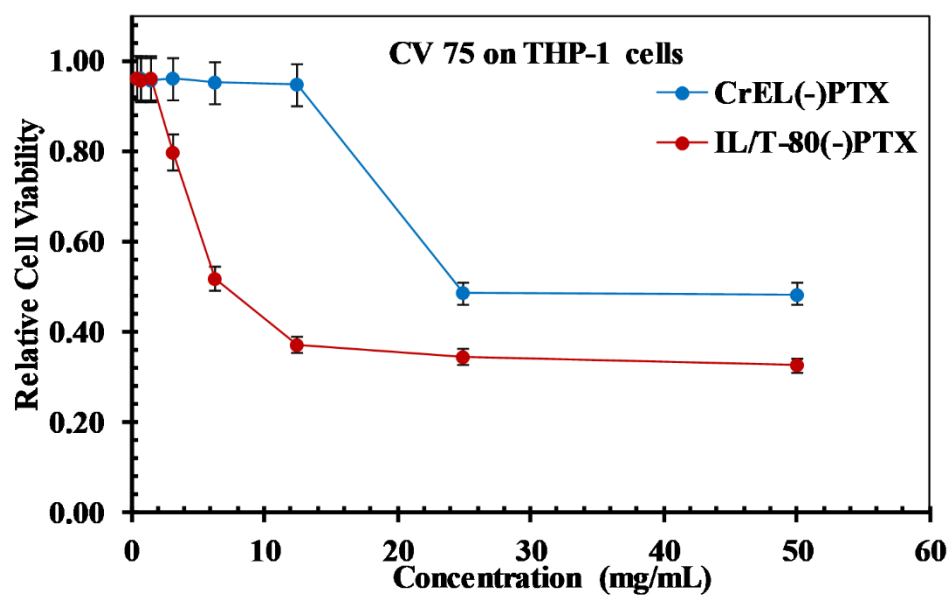


Figure 2-S5. Cell viability of THP-1 cells treated with IL/T-80(-) PTX or CrEL (-) PTX for determination of CV75.

[Ch][Gly] IL: ^1H -NMR (400 MHz, METHANOL- D_4) δ 4.88 (q, $J = 14.9$ Hz, 5H), 3.96–4.03 (m, 2H), 3.43–3.53 (m, 2H), 3.20 (d, $J = 8.2$ Hz, 9H), 3.14 (d, $J = 6.4$ Hz, 2H).

Elemental Analysis (%). Estimated: C 47.159, H 11.331, N 15.718; obtained: C 47.167, H 11.306, N 15.679. Yield: 87.6% based on the weight of choline chloride. Water content (Karl Fischer): 0.282% (w/w).

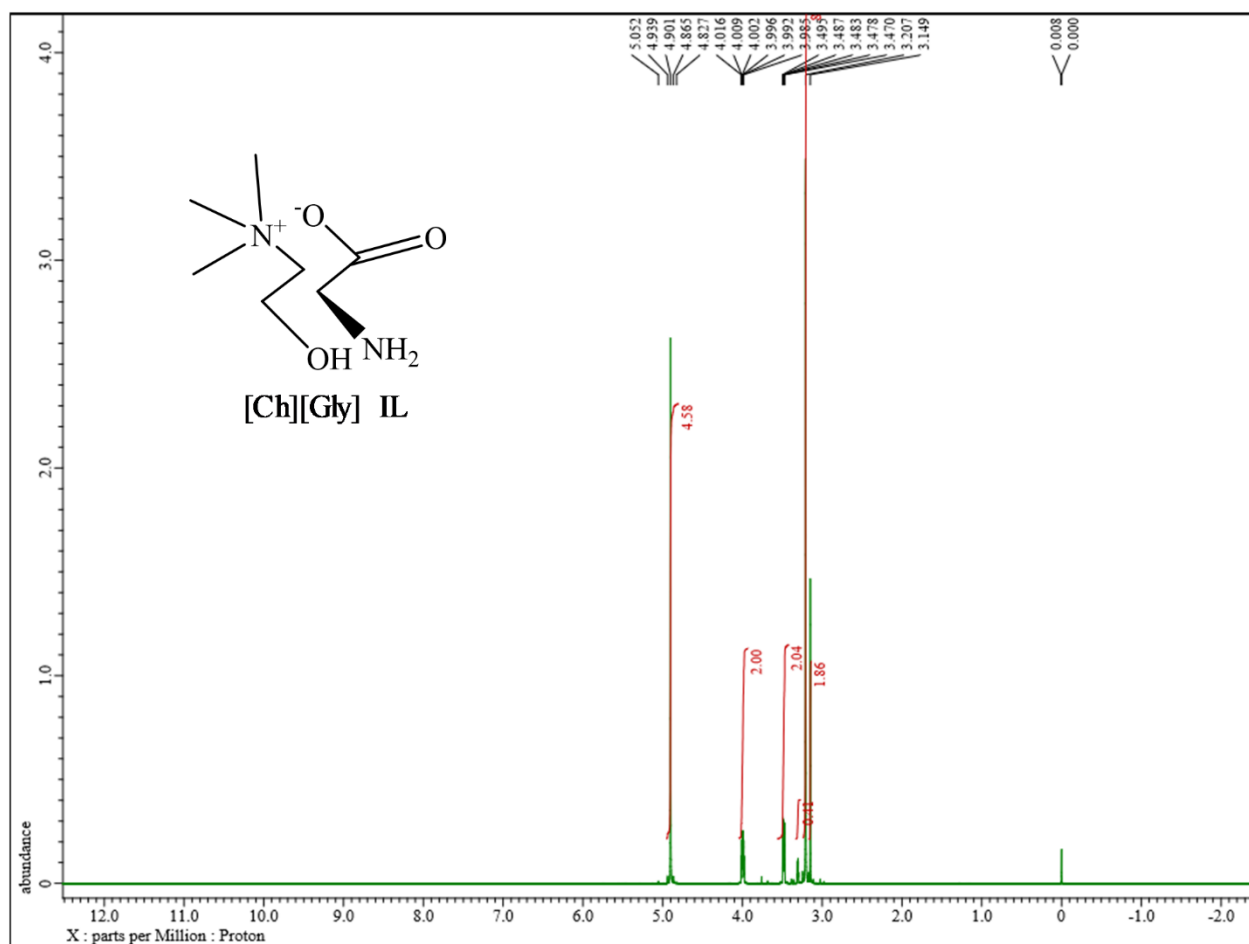


Figure 2-S6. ^1H NMR spectrum of [Ch][Gly] IL

[Ch][Ala] IL: ^1H -NMR (400 MHz, METHANOL- D_4) δ 4.90 (t, $J = 14.9$ Hz, 4H), 3.98–4.01 (m, 2H), 3.47–3.49 (m, 2H), 3.25–3.31 (m, 2H), 3.19 (d, $J = 15.1$ Hz, 9H), 1.27 (d, $J = 7.3$ Hz, 3H).

Elemental Analysis (%). Estimated: C 49.964, H 11.555, N 14.614; obtained: C 49.994, H 11.407, N 14.614. Yield: 85.5% based on the weight of choline chloride. Water content (Karl Fischer): 0.248% (w/w).

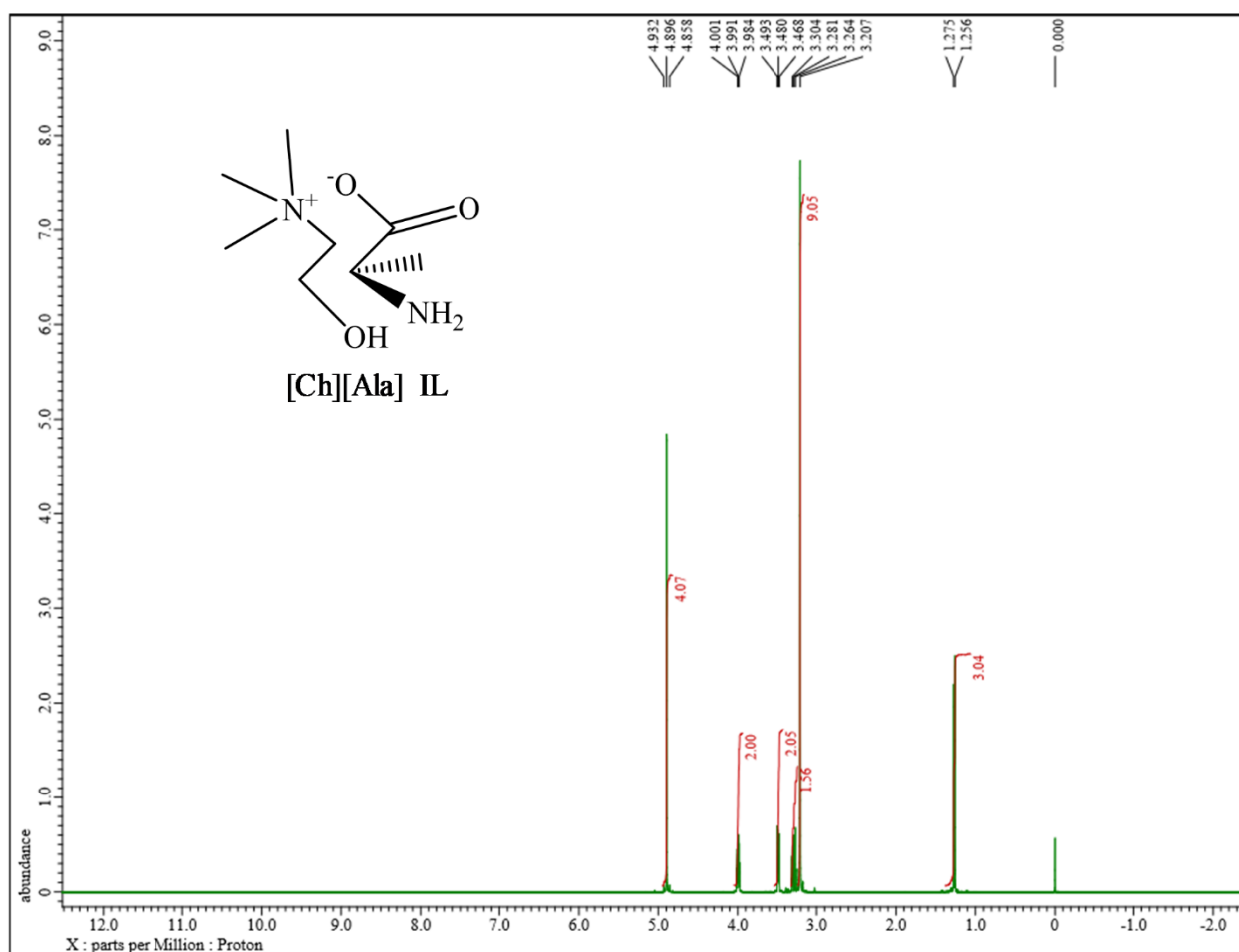


Figure 2-S7. ^1H NMR spectrum of [Ch][Ala] IL

[Ch][Ser] IL: ^1H -NMR (400 MHz, DMSO- D_6) δ 3.84 (d, J = 2.7 Hz, 2H), 3.41 (dd, J = 5.1, 3.5 Hz, 2H), 3.30–3.34 (m, 2H), 3.13 (d, J = 9.6 Hz, 9H), 2.93 (s, 1H), 2.50 (d, J = 1.8 Hz, 2H).

Elemental Analysis (%). Estimated: C 46.126, H 10.667, N 13.452; obtained: C 46.014, H 10.614, N 13.369. Yield: 87.4% based on the weight of choline chloride. Water content (Karl Fischer): 0.197% (w/w).

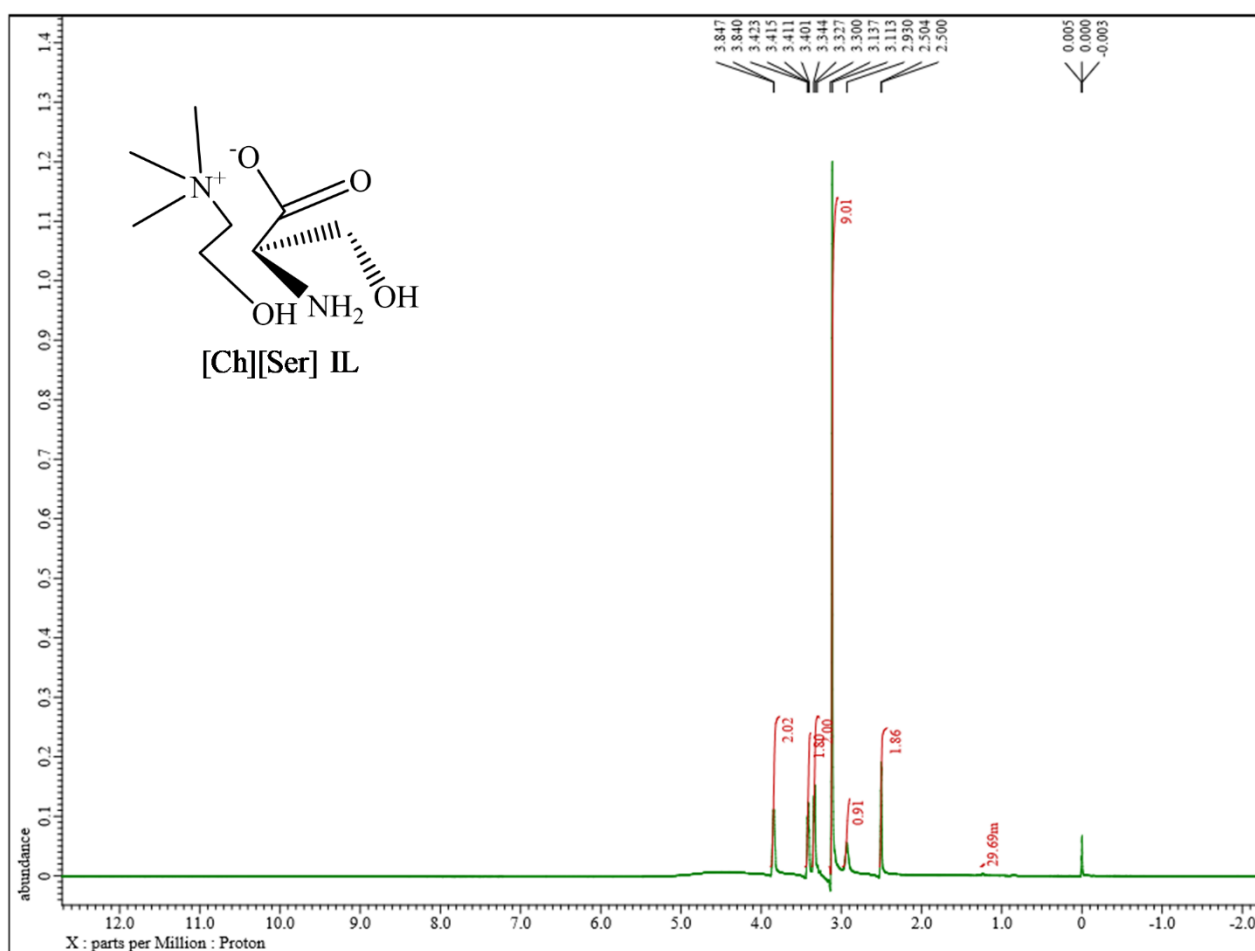


Figure 2-S8. ^1H NMR spectrum of [Ch][Ser] IL

[Ch][Pro] IL: ^1H -NMR (400 MHz, METHANOL- D_4) δ 4.91 (s, 8H), 3.98–4.01 (m, 3H), 3.64 (dd, J = 8.5, 6.2 Hz, 2H), 3.47–3.49 (m, 3H), 3.30–3.31 (m, 2H), 3.21 (s, 11H), 2.88–2.94 (m, 2H), 2.13–2.20 (m, 2H), 1.87–1.93 (m, 2H), 1.78–1.84 (m, 3H).

Elemental Analysis (%). Estimated: C 55.006, H 11.102, N 12.833; obtained: C 55.214, H 11.327, N 12.746. Yield: 88.7% based on the weight of choline chloride. Water content (Karl Fischer): 0.347% (w/w).

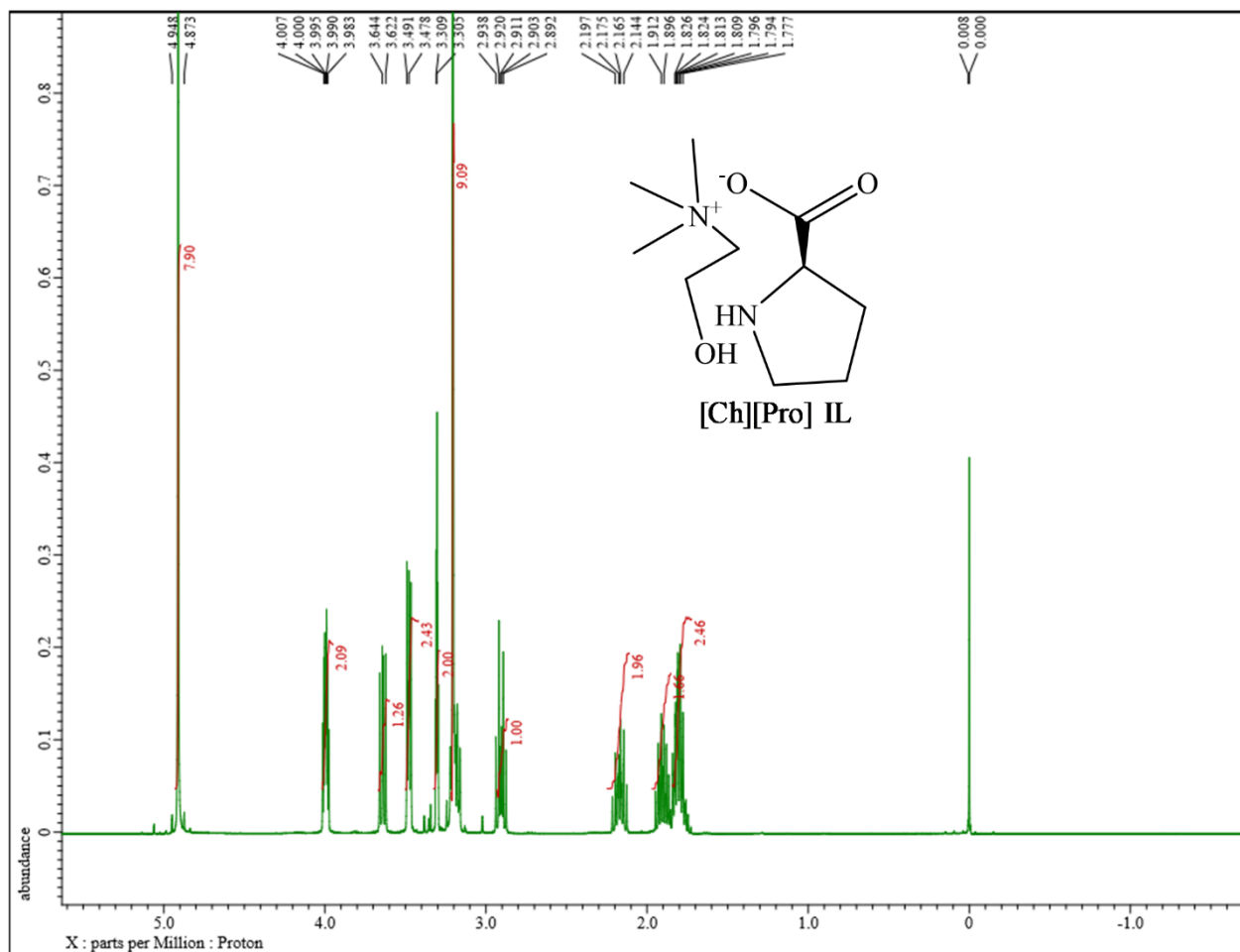


Figure 2-S9. ^1H NMR spectrum of [Ch][Pro] IL.

[Ch][Ile] IL: ^1H -NMR (400 MHz, METHANOL- D_4) δ 5.00 (t, J = 14.9 Hz, 6H), 3.98–4.02 (m, 2H), 3.60 (q, J = 7.0 Hz, 1H), 3.44–3.46 (m, 2H), 3.30–3.31 (m, 1H), 3.20 (t, J = 15.1 Hz, 9H), 1.7 (t, J = 7.1 Hz, 2H), 0.83–0.96 (m, 1H).

Elemental Analysis (%). Estimated: C 56.363, H 12.065, N 11.954; obtained: C 56.417, H 12.128, N 11.916. Yield: 87.8% based on the weight of choline chloride. Water content (Karl Fischer): 0.256% (w/w).

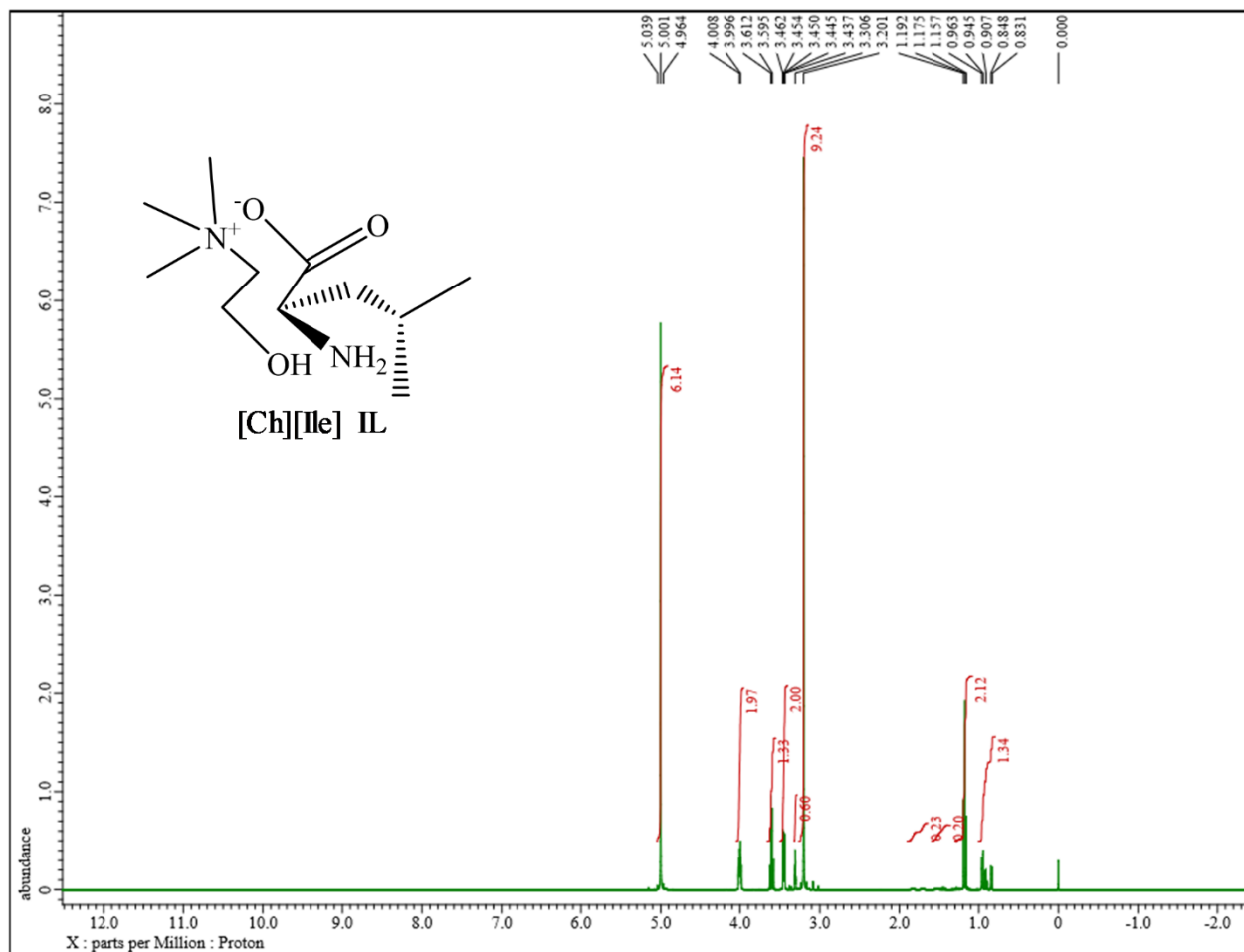
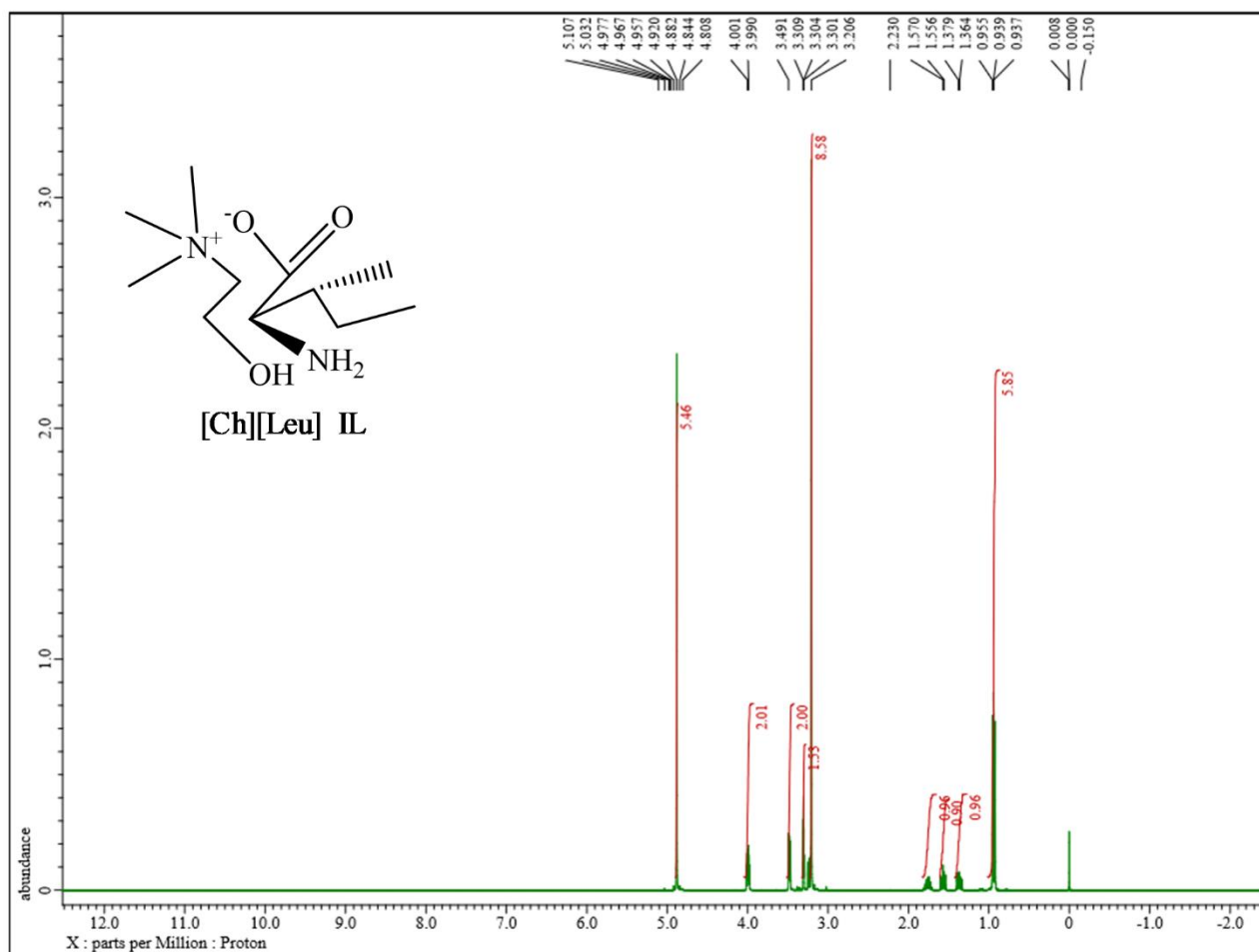


Figure 2-S10. ^1H NMR spectrum of [Ch][Ile] IL.

[Ch][Leu] IL: ^1H -NMR (400 MHz, METHANOL- D_4) δ 4.88 (s, 5H), 3.94–4.05 (m, 2H), 3.43–3.52 (m, 2H), 3.28–3.32 (m, 2H), 3.21 (d, $J = 5.0$ Hz, 9H), 1.69–1.82 (m, 1H), 1.50–1.60 (m, 1H), 1.29–1.40 (m, 1H), 0.88–1.01 (m, 6H).

Elemental Analysis (%). Estimated: C 56.363, H 12.065, N 11.954; obtained: C 56.504, H 12.107, N 11.917. Yield: 85.8% based on the weight of choline chloride. Water content (Karl Fischer): 0.274% (w/w).



Taure 2-S11. ¹H NMR spectrum of [Ch][Leu] IL.

[Ch][Phe] IL: ¹H-NMR (400 MHz, METHANOL-D₄) δ 7.26–7.28 (m, 3H), 7.18 (s, 1H), 4.89 (s, 4H), 3.96–4.00 (m, 2H), 3.44–3.48 (m, 3H), 3.30–3.31 (m, 1H), 3.19 (s, 9H), 3.11 (dd, J = 13.5, 4.8 Hz, 1H), 2.77 (dd, J = 13.3, 8.2 Hz, 1H).

Elemental Analysis (%). Estimated: C 62.645, H 9.784, N 10.440; obtained: C 62.498, H 9.746, N 10.417. Yield: 88.7% based on the weight of choline chloride. Water content (Karl Fischer): 0.267% (w/w).

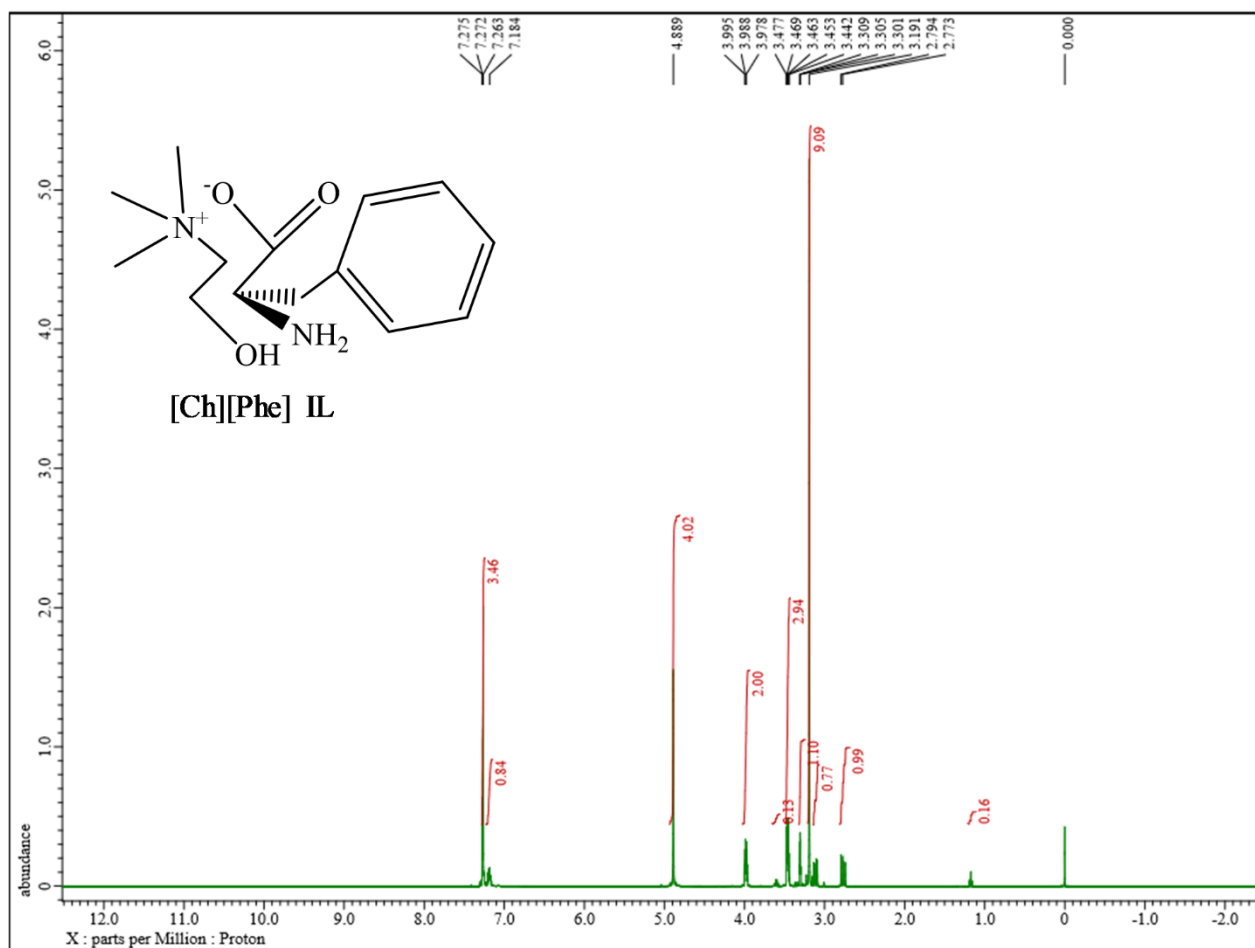


Figure 2-S12. ¹H NMR spectrum of [Ch][Phe] IL.

References

- (1) G. I. Kingston, D. Taxol: The Chemistry and Structure-Activity Relationships of a Novel Anticancer Agent. *TIBTECH(JUNE)* **1994**, 12, 222–227.
- (2) Hwu, J. R.; Lin, Y. S.; Josephrajan, T.; Hsu, M. H.; Cheng, F. Y.; Yeh, C. S.; Su, W. C.; Shieh, D. Bin. Targeted Paclitaxel by Conjugation to Iron Oxide and Gold Nanoparticles. *J. Am. Chem. Soc.* **2009**, 131 (1), 66–68.
- (3) Amos, L. A.; Löwe, J. How Taxol® Stabilises Microtubule Structure. *Chemistry and Biology*. 1999.
- (4) Khmelnitsky, Y. L.; Budde, C.; Arnold, J. M.; Usyatinsky, A.; Clark, D. S.; Dordick, J. S. Synthesis of Water-Soluble Paclitaxel Derivatives by Enzymatic Acylation [10]. *J. Am. Chem. Soc.* **1997**, 119 (47), 11554–11555.
- (5) Singla, A. K.; Garg, A.; Aggarwal, D. Paclitaxel and Its Formulations. *Int. J. Pharm.* **2002**, 235 (1–2), 179–192.
- (6) Gelderblom, H.; Verweij, J.; Nooter, K.; Sparreboom, A. Cremophor EL: The Drawbacks and Advantages of Vehicle Selection for Drug Formulation. *Eur. J. Cancer* **2001**, 37 (13), 1590–1598.
- (7) Moreno-Aspitia, A.; Perez, E. A. Nanoparticle Albumin-Bound Paclitaxel (ABI-007): A Newer Taxane Alternative in Breast Cancer. *Future Oncol.* **2005**, 1 (6), 755–762.
- (8) Gibson, J. D.; Khanal, B. P.; Zubarev, E. R. Paclitaxel-Functionalized Gold Nanoparticles. *J. Am. Chem. Soc.* **2007**, 129 (37), 11653–11661.
- (9) Huang, G.; Zang, B.; Wang, X.; Liu, G.; Zhao, J. Encapsulated Paclitaxel Nanoparticles Exhibit Enhanced Anti-Tumor Efficacy in A549 Non-Small Lung Cancer Cells. *Acta Biochim. Biophys. Sin. (Shanghai)*. **2015**, 47 (12), 981–987.
- (10) Fracasso, P. M.; Picus, J.; Wildi, J. D.; Goodner, S. A.; Creekmore, A. N.; Gao, F.; Govindan, R.; Ellis, M. J.; Tan, B. R.; Linette, G. P.; et al. Phase 1 and Pharmacokinetic Study of Weekly Docosahexaenoic Acid-Paclitaxel, Taxoprexin®, in Resistant Solid Tumor Malignancies. *Cancer Chemother. Pharmacol.* **2009**, 63 (3), 451–458.
- (11) Li, C.; Price, J. E.; Milas, L.; Hunter, N. R.; Ke, S.; Yu, D. F.; Charnsangavej, C.; Wallace, S. Antitumor Activity of Poly(L-Glutamic Acid)-Paclitaxel on Syngeneic and Xenografted Tumors. *Clin. Cancer Res.* **1999**, 5 (4), 891–897.
- (12) Régina, A.; Demeule, M.; Ché, C.; Lavallée, I.; Poirier, J.; Gabathuler, R.; Béliveau, R.; Castaigne, J. P. Antitumour Activity of ANG1005, a Conjugate between Paclitaxel and the New Brain Delivery Vector Angiopep-2. *Br. J. Pharmacol.* **2008**, 155 (2), 185–197.
- (13) Yang, T.; Choi, M. K.; Cui, F. De; Lee, S. J.; Chung, S. J.; Shim, C. K.; Kim, D. D. Antitumor Effect of Paclitaxel-Loaded PEGylated Immunoliposomes against Human Breast Cancer Cells. *Pharm. Res.* **2007**, 24 (12), 2402–2411.
- (14) Zhao, L.; Ye, Y.; Li, J.; Wei, Y. Preparation and the In-Vivo Evaluation of Paclitaxel Liposomes for Lung Targeting Delivery in Dogs. *J. Pharm. Pharmacol.* **2011**, 63 (1), 80–86.
- (15) Shi, Y.; Van Der Meel, R.; Theek, B.; Oude Blenke, E.; Pieters, E. H. E.; Fens, M. H.

- A. M.; Ehling, J.; Schiffelers, R. M.; Storm, G.; Van Nostrum, C. F.; et al. Complete Regression of Xenograft Tumors upon Targeted Delivery of Paclitaxel via π - π Stacking Stabilized Polymeric Micelles. *ACS Nano* **2015**, 9 (4), 3740–3752.
- (16) Egorova, K. S.; Gordeev, E. G.; Ananikov, V. P.; Zelinsky, N. D. Biological Activity of Ionic Liquids and Their Application in Pharmaceuticals and Medicine. *Chem. Rev.* **2017**, 117 (10), 7132–7189.
 - (17) Mehnert, C. P.; Cook, R. a; Dispenziere, N. C.; Afeworki, M. Supported Ionic Liquid Catalysis--a New Concept for Homogeneous Hydroformylation Catalysis. *J. Am. Chem. Soc.* **2002**, 124 (44), 12932–12933.
 - (18) Wasserscheid, P.; Keim, W. Ionic Liquids—New “Solutions” for Transition Metal Catalysis. *Angew. Chemie* **2000**, 39 (21), 3772–3789.
 - (19) Sasi, R.; Sarojam, S.; Devaki, S. J. High Performing Biobased Ionic Liquid Crystal Electrolytes for Supercapacitors. *ACS Sustain. Chem. Eng.* **2016**, 4 (6), 3535–3543.
 - (20) Moniruzzaman, M.; Goto, M. Ionic Liquids: Future Solvents and Reagents for Pharmaceuticals. *J. Chem. Eng. Japan* **2011**, 44 (6), 370–381.
 - (21) Ferraz, R.; Branco, L. C.; Prudêncio, C.; Noronha, J. P.; Petrovski, Ž. Ionic Liquids as Active Pharmaceutical Ingredients. *ChemMedChem* **2011**, 6 (6), 975–985.
 - (22) Eastoe, J.; Gold, S.; Rogers, S. E.; Paul, A.; Welton, T.; Heenan, R. K.; Grillo, I. Ionic Liquid-in-Oil Microemulsions. *J. Am. Chem. Soc.* **2005**, 127 (20), 7302–7303.
 - (23) Araki, S.; Wakabayashi, R.; Moniruzzaman, M.; Kamiya, N.; Goto, M. Ionic Liquid-Mediated Transcutaneous Protein Delivery with Solid-in-Oil Nanodispersions. *Med. Chem. Commun.* **2015**, 6 (12), 2124–2128.
 - (24) Goindi, S.; Arora, P.; Kumar, N.; Puri, A. Development of Novel Ionic Liquid-Based Microemulsion Formulation for Dermal Delivery of 5-Fluorouracil. *AAPS PharmSciTech* **2014**, 15 (4), 810–821.
 - (25) Moniruzzaman, M.; Tamura, M.; Tahara, Y.; Kamiya, N.; Goto, M. Ionic Liquid-in-Oil Microemulsion as a Potential Carrier of Sparingly Soluble Drug: Characterization and Cytotoxicity Evaluation. *Int. J. Pharm.* **2010**, 400 (1–2), 243–250.
 - (26) Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. Dissolution of Cellulose with Ionic Liquids. *J. Am. Chem. Soc.* **2002**, 124 (18), 4974–4975.
 - (27) Kim, S. Y.; Hwang, J. Y.; Seo, J. W.; Shin, U. S. Production of CNT-Taxol-Embedded PCL Microspheres Using an Ammonium-Based Room Temperature Ionic Liquid: As a Sustained Drug Delivery System. *J. Colloid Interface Sci.* **2015**, 442, 147–153.
 - (28) Tao, D. J.; Cheng, Z.; Chen, F. F.; Li, Z. M.; Hu, N.; Chen, X. S. Synthesis and Thermophysical Properties of Biocompatible Cholinium-Based Amino Acid Ionic Liquids. *J. Chem. Eng. Data* **2013**, 58 (6), 1542–1548.
 - (29) Liu, Q.-P.; Hou, X.-D.; Li, N.; Zong, M.-H. Ionic Liquids from Renewable Biomaterials: Synthesis, Characterization and Application in the Pretreatment of Biomass. *Green Chem.* **2012**, 14 (2), 304–307.
 - (30) Kesarwani, P.; Tekade, R. K.; Jain, N. K. Spectrophotometric Estimation of Paclitaxel. *Int. J. Adv. Pharm. Sci.* **2011**, 2 (1), 29–32.

- (31) Wu, Z.; Tucker, I. G.; Razzak, M.; Medlicott, N. J. An *in Vitro* Kinetic Method for Detection of Precipitation of Poorly Soluble Drugs. *Int. J. Pharm.* **2005**, *304* (1–2), 1–3.
- (32) Ashikaga, T.; Sakaguchi, H.; Okamoto, K.; Mizuno, M.; Sato, J.; Yamada, T.; Yoshida, M.; Ota, N.; Hasegawa, S.; Kodama, T.; et al. Assessment of the Human Cell Line Activation Test (h-CLAT) for Skin Sensitization; Results of the First Japanese Inter-Laboratory Study. *Aatex* **2008**, *13* (1), 27–35.
- (33) Nukada, Y.; Ashikaga, T.; Miyazawa, M.; Hirota, M.; Sakaguchi, H.; Sasa, H.; Nishiyama, N. Prediction of Skin Sensitization Potency of Chemicals by Human Cell Line Activation Test (h-CLAT) and an Attempt at Classifying Skin Sensitization Potency. *Toxicol. Vitro.* **2012**, *26* (7), 1150–1160.
- (34) Hou, X.-D. D.; Liu, Q.-P. P.; Smith, T. J.; Li, N.; Zong, M.-H. H. Evaluation of Toxicity and Biodegradability of Cholinium Amino Acids Ionic Liquids. *PLoS One* **2013**, *8* (3), e59145.
- (35) De Santis, S.; Masci, G.; Casciotta, F.; Caminiti, R.; Scarpellini, E.; Campetella, M.; Gontrani, L. Cholinium-Amino Acid Based Ionic Liquids: A New Method of Synthesis and Physico-Chemical Characterization. *Phys. Chem. Chem. Phys.* **2015**, *17* (32), 20687–20698.
- (36) Abe, M.; Kuroda, K.; Sato, D.; Kunitura, H.; Ohno, H. Effects of Polarity, Hydrophobicity, and Density of Ionic Liquids on Cellulose Solubility. *Phys. Chem. Chem. Phys.* **2015**, *17* (48), 32276–32282.
- (37) Fukaya, Y.; Sugimoto, A.; Ohno, H. Superior Solubility of Polysaccharides in Low Viscosity, Polar and Halogen-Free 1,3-Dialkylimidazolium Formates. *Biomacromolecules* **2006**, *7* (12), 3295–3297.
- (38) Moes, J.; Koolen, S.; Huitema, A.; Schellens, J.; Beijnen, J.; Nuijen, B. Development of an Oral Solid Dispersion Formulation for Use in Low-Dose Metronomic Chemotherapy of Paclitaxel. *Eur. J. Pharm. Biopharm.* **2013**, *83* (1), 87–94.
- (39) Marmur, A. Dissolution and Self-Assembly: The Solvophobic/Hydrophobic Effect. *J. Am. Chem. Soc.* **2000**, *122* (9), 2120–2121.
- (40) Lu, B.; Xu, A.; Wang, J. Cation Does Matter: How Cationic Structure Affects the Dissolution of Cellulose in Ionic Liquids. *Green Chem.* **2014**, *16* (3), 1326–1335.
- (41) Merisko-Liversidge, E.; Liversidge, G. G.; Cooper, E. R. Nanosizing: A Formulation Approach for Poorly-Water-Soluble Compounds. *European Journal of Pharmaceutical Sciences*. 2003, pp 113–120.
- (42) Petkovic, M.; Ferguson, J. L.; Gunaratne, H. Q. N.; Ferreira, R.; Leitão, M. C.; Seddon, K. R.; Rebelo, L. P. N.; Pereira, C. S. Novel Biocompatible Cholinium-Based Ionic Liquids—Toxicity and Biodegradability. *Green Chem.* **2010**, *12* (4), 643.
- (43) Galbiati, V.; Papale, A.; Kummer, E.; Corsini, E. *In Vitro* Models to Evaluate Drug-Induced Hypersensitivity: Potential Test Based on Activation of Dendritic Cells. *Front. Pharmacol.* **2016**, *7*, 1–10.
- (44) Sakaguchi, H.; Ashikaga, T.; Miyazawa, M.; Yoshida, Y.; Ito, Y.; Yoneyama, K.; Hirota, M.; Itagaki, H.; Toyoda, H.; Suzuki, H. Development of an *in Vitro* Skin Sensitization Test Using Human Cell Lines; Human Cell Line Activation Test (h-CLAT). II. An Inter-

Laboratory Study of the h-CLAT. *Toxicol. In Vitro* **2006**, 20 (5), 774–784.

- (45) Sakaguchi, H.; Ashikaga, T.; Miyazawa, M.; Kosaka, N.; Ito, Y.; Yoneyama, K.; Sono, S.; Itagaki, H.; Toyoda, H.; Suzuki, H. The Relationship between CD86/CD54 Expression and THP-1 Cell Viability in an *in Vitro* Skin Sensitization Test - Human Cell Line Activation Test (h-CLAT). *Cell Biol. Toxicol.* **2009**, 25 (2), 109–126.

Nomenclature and Abbreviations for Chapter-2

Abbreviation	Name/description
AA	Amino Acid
Ala	Alanine
API	Active Pharmaceutical Ingredients
FBS	Fetal Bovine Serum
CCK-8	Cell Counting Kit-8
CD	Cluster of Differentiation
[Ch][AA]	Choline Amino Acid Ionic Liquid
CNT	Carbon-nanotube
CrEL	Cremophor EL, ethoxylated castor oil
DLS	Dynamic Light Scattering
DNCB	2,4-Dinitrochlorobenzene
EC	Estimated Concentration
EtOH	Ethanol (dehydrated)
FACS	Fc Receptor Blocking Solution
Gly	Glycine
h-CLAT	Human Cell Line Activation Test
HeLa	Human Cervical Cancer Cells
HPLC	High-Performance Liquid Chromatography
HSPC	Phospholipon 90H
IgG	Immunoglobulin G
IL	Ionic Liquid
Ile	Isoleucine
JRCB bank	Japanese Cancer Research Resources Bank
KF	Karl Fisher

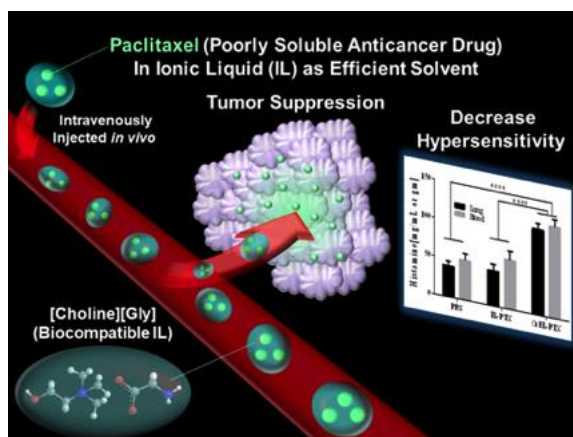
Leu	Leucine
ME	Microviscometer
MEM	Minimum Essential Media
MFI	Geometric Mean Fluorescence Intensity
NIHS	National Institute of Hygienic Health Sciences
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PCL	Polycaprolactone
PEG	Polyethylene glycol
PI	Propidium Iodide
Phe	Phenyl Alanine
Pro	Proline
PTX	Paclitaxel
RFI	Relative Fluorescent Intensity
RPMI	Roswell Park Memorial Institute Medium
Ser	Serine
T-80	Polyoxyethylene 20 sorbitan monooleate
THP-1	Human Monocyte Cell Line
TOMAC	Trioctylmethyammonium Chloride

CHAPTER-3

IN VIVO BIOCOMPATIBILITY, PHARMACOKINETICS, ANTITUMOR EFFICACY, AND HYPERSENSITIVITY EVALUATION OF IONIC LIQUID-MEDIATED PACLITAXEL FORMULATIONS

3.1 Abstract

In order to prevent common hypersensitivity reactions to paclitaxel injections (Taxol), we previously reported an ionic liquid-mediated paclitaxel (IL-PTX) formulation with small particle size and narrow size distribution. The preliminary work showed high PTX solubility in the IL, and the formulation demonstrated similar antitumor activity to Taxol, while inducing a smaller hypersensitivity effect in *in vitro* cell experiments. In this study, the stability of the IL-PTX formulation was monitored by quantitative HPLC analysis, which showed that IL-PTX was more stable at 4 °C than at room temperature. The *in vivo* study showed that the IL-PTX formulation could be used in a therapeutic application as a biocompatible component of a drug delivery system. To assess the *in-vivo* biocompatibility, IL or IL-mediated formulations were administered intravenously by maintaining physiological buffered conditions (neutral pH and isotonic salt concentration). From *in vivo* pharmacokinetics data, the IL-PTX formulation was found to have a similar systemic circulation time and slower elimination rate compared to cremophor EL mediated paclitaxel (CrEL-PTX). Furthermore, *in vivo* antitumor and hypersensitivity experiments in C57BL/6 mice revealed that IL-PTX had similar antitumor activity to CrEL-PTX, but a significantly smaller hypersensitivity effect compared with CrEL-PTX. Therefore, the IL-mediated formulation has potential to be an effective and safe drug delivery system for PTX.



Graphical Presentation of *In-Vivo* Evaluation of IL-mediated Formulation.

3.2 Introduction

Paclitaxel (PTX) has long been considered an excellent naturally derived chemotherapeutic agent against different types of cancer such as ovarian, lung, and breast cancer, as well as acute leukemia, head and neck carcinoma, and AIDS-related Kaposi's sarcoma.¹⁻³ However, low aqueous solubility (<4.0 µg/mL) and poor permeability across biological membranes are the main barriers to its widespread application.^{4,5} The current most commonly administered clinical formulation (Taxol, Bristol-Myers Squibb, New York, NY, USA) consists of a vehicle system of Cremophor EL (polyethoxylated castor oil, CrEL) and dehydrated ethanol (EtOH) in a 50:50 ratio (1:1, v/v) to enhance its solubility and stability in water/saline(0.9% NaCl)/dextrose(5.0% glucose) solution during intravenous (IV) infusion/injection.⁶ The use of a large amount of CrEL in this formulation is often associated with various side effects such as hypersensitivity (minor reaction: flushing and rash in 41%–44% of patients, and major reaction: potentially life threatening in 1.5%–3% of patients), abnormal lipoproteins, aggregation of erythrocytes, neuropathy, vasodilation, dyspnea, and hypotension.⁷⁻¹⁰ As a preventive action, continuous administration of antihistamines and corticosteroids at high doses is required throughout the treatment/pretreatment, resulting in significant patient noncompliance.¹¹ Numerous strategies have been investigated for developing drug delivery systems without CrEL, including formation of albumin, nanoparticle, emulsion, prodrug, liposome, porous particle, polymeric micelle, and ionic liquid mediated microspheres of PTX.¹¹⁻¹⁶ Ionic Liquids (ILs) are a class of organic salts with unusual physico-chemical and biological characteristics compared with conventional organic solvents.^{11-13,17,18} To date, one formulation (Abraxane: Conjugated albumin-PTX particle for injection) has had approval from the FDA for clinical application and several promising candidates are in preclinical/clinical trials.¹⁹⁻²¹

In our previous study, an IL-based PTX formulation was developed in which a mixture of IL, EtOH, and Tween-80 (T-80) was used to solubilize and stabilize PTX in aqueous solution. PTX solubility was found to be significantly enhanced in IL compared with water. Furthermore, the IL-mediated PTX formulation (IL-PTX) exhibited small particle size and a narrow size distribution, lower cytotoxicity, and similar antitumor activity to Taxol against HeLa cells in *in vitro* experiments. Moreover, the hypersensitivity effects of the IL-based formulation were found to be significantly reduced compared with CrEL-based formulations in a human leukemia cell line, THP-1.²² But it is prerequisite to evaluate the biocompatibility of an IL to be used in drug delivery system. As a follow-up to that study, further investigation of the IL-

based PTX formulation is reported in this paper to evaluate the *in vivo* biocompatibility, pharmacokinetics, antitumor activity, and hypersensitivity in a mouse model with CrEL-mediated PTX (CrEL-PTX or Taxol) as a reference.

3.3 Experimental

3.3.1 Materials and Methods

3.3.2 Materials

Paclitaxel (PTX) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cremophor EL (CrEL) was purchased from Fluka Biochemika, Germany. Choline chloride, glycine and anhydrous ethanol (EtOH) were procured from Chameleon Guaranteed Reagent, Japan. HPLC grade methanol, acetonitrile, and silver oxide were purchased from Wako Pure Chemical Industries, Ltd., Japan. Polysorbate 80 (Tween-80, T-80) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade. A Milli-Q purification system from Millipore (Molsheim, France) was used to prepare de-ionized and ultrapure water throughout the experiments. Hank's balanced salt solution (HBSS) medium, D-phosphate buffered saline {(–) D-PBS}, fetal bovine serum (FBS), antibiotic-antimycotic (Streptomycin sulfate and Penicillin G. Sodium, AA), and Opti-MEM were purchased from Gibco, Life Technologies Corp., USA. Roswell Park Memorial Institute (RPMI) medium and trypsin/1,2-ethylenediaminetetraacetic acid (EDTA) were purchased from Nacalai Tesque Inc., Japan. Cell counting kit-8 (WST-kit) was purchased from Dojindo Co., Japan. Cell Culture Dishes and 96-well plates were purchased from CELLSTAR of Greiner Bio-One GmbH, Germany.

3.3.3 Animals and Cell Lines

Female C57BL/6NJc1 (C57BL/6) mice (8 weeks old, 20 ± 2 g) were obtained from CLEA Japan, Inc. All of the pathogen-free animals were housed under natural light/dark conditions (room temperature: $25 \pm 2^\circ\text{C}$, relative humidity: $60 \pm 10\%$) for at least 1 week before the experiments. Animals were cared for and handled according to the Animal Ethics Committee of Kyushu University, Japan.

A mouse skin melanoma cell line (B16F10, strain: C57BL/6J) was obtained from the RIKEN cell bank.

3.3.4 Chromatographic Conditions

The concentrations of PTX in different samples (stability and plasma/blood) were estimated using a HPLC system consisting of a pump (PU-4180), a photo diode array (PDA) detector (MD-4010), an auto sampler (AS-4050), and a column oven controller (CO-4060) with an interface box (LC-NetII/ADC) and a bottle stand (BS-4000-1) (JASCO International Co. Ltd., Hachioji, Tokyo 193-0835 Japan). The mobile phase consisted of water, methanol, and acetonitrile at a ratio of 45/5/50 (v/v/v, %) and was delivered at a flow rate of 1.0 mL/min. The chromatographic separation was performed on an Inertsil ODS-3 column (4.6×250 mm, 5 µm, GL Sciences Inc., Shinjuku-ku, Tokyo, 163-1130, Japan) at a wavelength of 228 nm. The peak areas for PTX in various samples were recorded, and the concentrations of PTX in unknown samples were estimated using a calibration curve derived from standard samples.

3.3.5 Stability of the IL-mediated PTX Formulation

The IL-mediated PTX formulation (IL-PTX, 0.5 mg/mL PTX) used in the experiments was prepared in our laboratory according to a published procedure.²³ In this study, all experiments were performed using [Choline][Glycine] as the IL. Briefly, 0.5 mg of PTX was dissolved in 100 µL of EtOH and then 50 µL of IL and 50 µL of T-80 were added. The final formulation was obtained by adding 800 µL of water and mixing well (Table 3-1). The solution was then stored in glass vials at room temperature (RT, 25 °C, away from direct sunlight) or under refrigeration at 4 °C. The PTX content of the initial sample was analyzed by HPLC, and tests were subsequently repeated at days 1, 3, 7, 15, and 30 of incubation. Briefly, 50 µL of IL-PTX solution was removed, diluted to 1 mL with mobile phase and mixed well by vortexing. Subsequently, 100 µL of that solution was further diluted to 500 µL using the same diluent and mixed well. The resulting solution was filtered through a 0.22 µm membrane filter and 10 µL of filtrate was injected into the HPLC system to determine the PTX content.

3.3.6 Intravenous (IV) Injection Safety Assessment

3.3.7 IV Administration of IL or IL-mediated Formulations

C57BL/6 mice were used to check the biocompatibility of IL or IL-mediated formulations by IV injection. Two types of IL formulation, IL_{only}[-]PTX and IL-[-]PTX as defined in Table 3-1, were used at different concentrations. Briefly, prepared IL_{only}[-]PTX and IL-[-]PTX were diluted with PBS (pH 7.0 ± 0.2) to obtain different solution concentrations as shown in Table 3-S1 and 3-S2, respectively. With the exception of the first sample of the IL_{only}[-]PTX-group, the pH of all samples was adjusted and maintained at ~7.0. The samples were filtered through a 0.22 µm sterile membrane filter and injected into the tail vein (100 µL/mouse). The rate of survival (% of survival) and weight variation (% of weight variation) of the surviving mice were monitored up to 4 weeks as a measurement of the immediate and long-term toxicological effect of IL on IV administration.

Table 3-1. Compositions of different formulations of IL and CrEL with/without PTX. IL-PTX and CrEL-PTX contained 0.5 mg/mL PTX at final concentration. The symbol (+) or (-) denotes as the presence/absence of particular material in the row.

Formulation	PTX	Solvent compositions in water (% w/v)			
		IL	T-80	EtOH	CrEL
IL _{only} [-]PTX	-	5	-	-	-
IL-[-]PTX	-	5	5	10	-
IL-PTX	+	5	5	10	-
CrEL-[-]PTX	-	-	-	10	10
CrEL-PTX	+	-	-	10	10

3.3.8 Intravenous Irritation Assessment

First, both the IL-PTX and CrEL-PTX formulations from Table 3-1 were diluted with PBS to give a concentration of 0.1 mg/mL of PTX. Subsequently, each mouse received a daily dose of 10 µg of PTX in 100 µL of either the IL-PTX or CrEL-PTX diluted formulations via the tail vein. An equivalent volume of PBS was injected in the same way as a control. Immediately after injection, the behavioral changes and clinical signs of hypersensitivity were observed for 1 h as described in Table 3-S3. The reaction at the injection site was also monitored up to 14 days post injection.

3.3.9 Pharmacokinetics Studies

C57BL/6 mice were randomly divided into two groups each consisting of five mice. All of the animals were fasted for 6 h prior to the experiments. First, freshly prepared IL-PTX and CrEL-PTX were diluted with PBS to a concentration of 0.1 mg/mL of PTX, where the IL concentration was fixed at 50.94 mM. The diluted formulations were filtered through 0.22 μ m sterile membrane filters and injected into the tail vein at a single dose of 2.5 mg/kg of PTX. Subsequently, 50 μ L of blood was collected at 10, 20, 40, 60, 90, 120, 180, and 240 min post injection. The PBS solution was injected in the same way as a control. The blood samples were then immediately processed for further analysis according to a previously published method with slight modification.²⁴ Briefly, an equal volume (w/w) of HPLC mobile phase was added to the blood samples, which were then vortexed and mixed well by sonication for 30 min. The samples were then centrifuged at 15000 rpm for 30 min, the supernatant was collected, filtered through a 0.22 μ m membrane filter and 25 μ L of the filtrate was injected into the HPLC-system for analysis.

3.3.10 *In Vivo* Antitumor Efficacy

Before progressing to the *in vivo* experiment, the antitumor activity of IL-PTX against B16F10 cells was evaluated using the WST assay method. Briefly, 5×10^3 cells/well in 96-well plates were incubated for 24 h at 37 °C in a 5 % CO₂ incubator (Espec Corp., BNS-110, Japan). The cells were then incubated with IL-PTX, IL-[-]PTX, CrEL-PTX, and CrEL-[-]PTX (as defined in Table 3-1) at a series of PTX concentrations for 24 h. The cell viability (%) of each group was determined using the WST assay method following a previously published procedure.²²

In the *in vivo* study, C57BL/6 mice were inoculated subcutaneously in the right flank with 100 μ L of a B16F10 cell suspension (1×10^6 cells/mL in HBSS buffer, pH 7.4) to develop a tumor mass. The experiment was carried out in three different categories.

Category-1: Four different formulations of IL and CrEL as described in Table 3-1 denoted IL-[-]PTX, IL-PTX, CrEL-[-]PTX, and CrEL-PTX were administered as single doses. PBS solution was used as a control. Upon appropriate dilution with PBS, all of the formulations were administrated into tumors on day 14 after inoculation when the average size of the tumor mass was approximately 355 ± 41 mm³.

Category-2: The experimental setup was similar to category-1, with tumor bearing mice receiving the first intratumoral dose at day 14 after B16F10 cell inoculation. Second and third doses were then administered at days 17 and 20.

Category-3: The IL-PTX formulation was intratumorally administrated at a different phase of tumor growth or B16F10 cell division after inoculation. Three different phases were considered in this category. The first was administration at day 3 after inoculation (after 4 cell cycle divisions), when the tumor lump was not visible (undeveloped stage). The second was administration at day 5 after inoculation when the tumor size reached approximately 25 mm³ (tumor lump was visible, developing stage). The third was administration at day 8 after inoculation when the tumor size reached approximately 100 mm³ (developed stage). At each stage, mice were divided randomly into control (PBS), IL-PTX, and CrEL-PTX groups with 5 mice in each group. Multi-dose administrations were carried out at every third cell division cycle (48~55 h) after the initial dose and continued until mice were euthanized.

In the antitumor experiment, both IL-PTX and CrEL-PTX or their placebo formulations were diluted with PBS. In each group, each administration was given at a dose of 2.5 mg/kg of PTX. Throughout the study, tumor volume was measured with calipers every day using the following formula: $V = (\text{major axis [mm]} \times (\text{minor axis [mm]})^2 \times 0.5$. The measurement of tumor volume was carried out until the day the mice were euthanized and the survival day was also recorded.

3.3.11 *In Vivo* Hypersensitivity Studies

IL-PTX and CrEL-PTX were freshly prepared (as described in Table 3-1) and diluted with PBS prior to use. PBS was used as a control and the doses (2.5 mg/kg) were administrated intravenously in a 10 mL/kg injection volume to the mice (6 mice per group). Signs of allergic reaction were observed and scored according to Table 3-S2. Blood samples were collected at 90 min after the single dose, and then injected mice were euthanized. Serum samples were prepared by centrifugation at 3000 rpm for 20 min and the serum complement split product (SC5b-9) and histamine contents were measured following the guidelines of the commercial ELISA kits (Histamine, Neogen Corp., USA and SC5b-9, Cosmo Bio Co., LTD., Tokyo, Japan). After the mice were euthanized, the lungs were removed as soon as possible and weighed to determine the organ/body ratio. The lung tissues were cooled, homogenized and tested for histamine using commercial ELISA kits with appropriate dilution.

3.3.12 Data Analysis and Statistics

All of the results were presented as means $\pm$ SD. The pharmacokinetic parameters were obtained by manual calculation based on theories and equations using Microsoft Excel (MS Office Program, 2016). Statistical moment theory was applied for the area under the curve (AUC) calculation. Comparisons between more than two groups were performed by one-way ANOVA analysis, followed by Tukey's multiple comparison test using GraphPad Prism software (Version 6.05). Two-way ANOVA followed by Dunnett's multiple comparison test was used for the analysis of histamine release in the blood and lung lesions. Pharmacokinetics data were analyzed by multiple t tests using Holm-Sidak method. Differences were considered to be significant at $p < 0.05$.

3.4 Results and Discussion

3.4.1 HPLC Analysis of Stability Samples

In order to analyze PTX in the IL-PTX and plasma samples, a HPLC approach was developed by adapting an established method.²⁵ Using this method, the retention time of PTX was found to be approximately 15.5 (14.5~17.0) min. Fig.3-S1a and 3-S1b present the chromatographs of standard PTX solution, IL-PTX, and all possible placebo formulations. The PTX peak was found to be specific and selective without any interference from the placebo. A linear calibration curve was established within the range 0.5–2.5 $\mu\text{g/mL}$ (correlation coefficient, $R^2 = 0.9987$) for analysis of PTX in the stability samples (Fig.3-S2a). The stability of the IL-PTX formulations was measured through the quantitative HPLC analysis of PTX stored in two different temperature conditions. No degradation peak (no extra peak in the chromatograph) was observed at up to 4 weeks during the stability study (Fig.3-S2b & 3-S2c). Fig.3-1 shows the PTX content retained in the IL-mediated formulations at two different temperatures (RT and 4 °C). At one week post storage, the mean PTX concentration was found to be ~ 91% and 96% of the initial concentration at RT and 4 °C, respectively. No precipitation was observed in the samples after one month of storage particularly at 4 °C, which showed a mean PTX concentration of >95% of the initial concentration. The mean PTX concentration in the RT samples was found to be unstable and to show slight decline. After four weeks of storage, the mean PTX concentration was ~88% of the initial concentration with the presence of small aggregates or particles. It was previously reported that the combination of IL, T-80, and EtOH might create a steric and ionic barrier to PTX by creating a hydrophobic pocket inhibiting the self-aggregation of PTX.²² After long storage times, this interaction might be

weakened at RT compared with at 4 °C, resulting in slowly release and precipitation of the PTX molecule. So, special care such as maintaining the refrigeration temperature (~ 4 °C) is an important parameter to keep the formulation stable for long term storage as well as transportation.

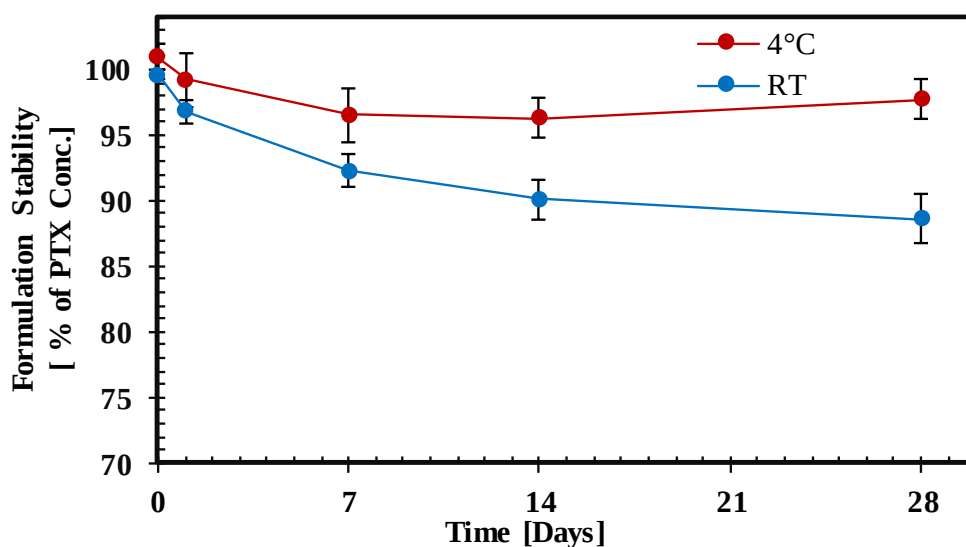


Figure 3-7. Stability of the IL-PTX formulation as a quantitative analysis by HPLC.

3.4.2 Intravenous (IV) Injection Safety Assessments

3.4.3 Administration of IL or IL-mediated Formulations

Biocompatibility is the foremost issue for materials that are to be used as vehicles in drug delivery systems. ILs or mixtures of ILs or/and IL-based solvents systems were reported to be used for solubilization of sparingly water soluble drugs.^{26–28} Here, we propose the use of an IL ([Choline][Glycine]) as an alternative solubilizing and stabilizing agent in combination with T-80 and ethanol. This IL was chosen to be a component of the vehicle because the cation, cholinium, derived from choline, is an essential micronutrient that can be completely metabolized as a result of having a polar hydroxyl group; and the glycine anion, is part of one of the most abundant classes of organic molecules in nature, and is considered nontoxic, biodegradable, and biocompatible.^{29,30} Furthermore, the use of T-80 and ethanol as solubilizing agents in pharmaceutical formulation have been widely accepted, especially to enhance the drug solubility and permeability across the biological membrane.^{31,32} The survival rate was 100 % when solutions containing IL concentrations < 1.0 % (around 56 mM) either in IL_{only}[-]PTX or IL-[-]PTX were used, confirming the biocompatibility. The solutions to be administrated

intravenously for therapeutic purpose should be isotonic and the pH must be in the physiological range ~7.4.

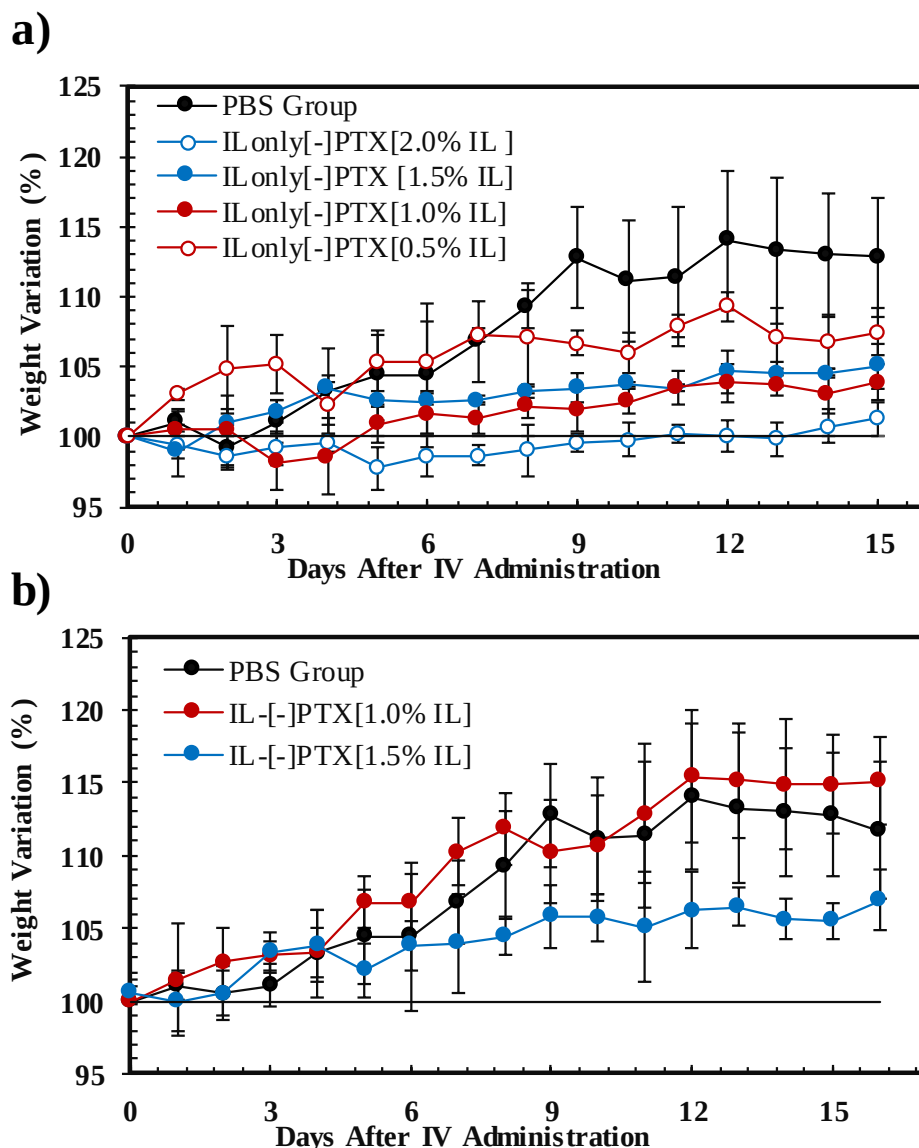


Figure 3-8. Percentage (%) weight variation as a measure of safety and toxicity after intravenous administration of (a) IL_{only}[-]PTX and (b) IL-[-]PTX as a single dose of 2.5 mg/kg of PTX in C57BL/6 mice. The straight line at 100 represents ‘no change in weight’. The percentage weight variation above the straight line was considered to indicate the healthy condition of the mice.

The total salt concentration in 1.0% IL solution was around 185 mM. This is the lowest salt concentration of IL solution (if IL considered as salt) compared with saline (0.9% NaCl solution, salt concentration: 154 mM) or PBS (salt concentration: 151.5 mM). As IL possesses

cationic and anionic domains the same as other salts of PBS solution or saline, this transmuted the isotonic PBS solution to hypertonic in the presence of IL.

Physical effects, such as loss of appetite or imbalanced movement as a consequence of body weight loss, can be important monitoring parameters to give initial indications of a chemical vehicle being toxic or non-biocompatible after administration. Fig.3-2a and 3-2b, show the percentage weight variation of the surviving mice after IV administration of different concentrations of IL_{only}[-]PTX or IL-[-]PTX solutions. The control group of mice, which was administrated PBS only, clearly showed an ascending trend in the weight variation curve, indicating a good safety profile. Almost similar patterns of ascending weight were also obtained for the IL_{only}[-]PTX and IL-[-]PTX formulations. In case of the 1.5% (84 mM) and 2.0% (112 mM) IL solutions, the mean weight variations were found to decrease after the initial administration but were restored and healthier indications were achieved within 2 weeks.

3.4.4 Intravenous Irritation Assessment

Single dose intravenous irritation was assessed in C57BL/6 mice for IL-PTX and CrEL-PTX. Behavioral changes were observed and the effects of hypersensitivity reactions were graded according to Table 3-S3.¹¹ All of the mice in the CrEL-PTX group were observed to have acute hypersensitivity reactions at 3–5 min after injection that recovered within 25–40 min. However, mice in the IL-PTX group showed much slighter responses. The presence of CrEL may have induced the hypersensitivity reactions for the CrEL-PTX group through complement activation, while the IL-PTX group showed milder responses. It has been reported that anti-histamine drugs such as dexamethasone and cimetidine can be premeditated to mitigate the hypersensitivity effects during CrEL-PTX treatment.¹¹ However, the IL reported here could be delivered as a component of the drug delivery system without the need for other drugs to mitigate side effects. In addition, the biocompatibility of the IL can be adjusted as desired by modifying/altering the cation and/or anion with alternatives from a wide range of biological sources.³³

3.4.5 Pharmacokinetics Study

In this study, the PTX in the plasma samples was completely separated during HPLC analysis and was quantitatively assayed using a linear standard calibration ranging from 100 ng/mL to 500 ng/mL with a correlation coefficient value of 0.9969 (Fig.3-S3a). The components of blood/plasma did not interfere with the detection of the PTX peak during HPLC analysis (Fig.3-S3b & 3-S3c). The plasma concentration time profile of the IL-PTX and CrEL-

PTX formulations after IV administration, and their corresponding pharmacokinetic (PK) parameters, are compared in Fig.3-3 and Table 3-2 for a single dose of 2.5 mg/kg of PTX in C57BL/6 mice.

Table 3-2. Pharmacokinetic parameters of PTX in mice after intravenous administration of IL-based PTX formulations at a dose of 1.0 mg/kg. Sample volume was 100 μ L per mouse. (Mean $\pm$ SD, n = 3), * p <0.05.

Parameters	CrEL-PTX	IL-PTX
V_d (mL)	8.5 ± 0.4	7.4 ± 0.4^{ns}
C_{max} (ng/mL)	1188 ± 61	$1354 \pm 92^*$
$t_{1/2}$ (min)	43.8 ± 4.6	45.3 ± 4.4^{ns}
CL (mL/min)	0.13 ± 0.01	0.11 ± 0.01^{ns}
AUC_{0-t} (ng.hr/mL)	1279 ± 199	$1496 \pm 93^*$
C_0 (ng/mL)	1180 ± 62	$1354 \pm 67^*$
Rate constant, k (min^{-1})	0.016 ± 0.001	0.015 ± 0.001^{ns}

^{ns} not significant

The PK profile was found to be fitted to a non-compartmental model. Both formulations showed similar plasma PK profile patterns, such as rapid distribution in the initial phase followed by a sustained circulation phase. In the first 60 min after dosing, a rapid decline of the distribution phase was observed. Both formulations showed maximum PTX concentrations in plasma within 5–8 min of injection. The calculated maximum concentration (C_{max}) and the bioavailability (AUC_{0-t}) of IL-PTX were found to be higher than for CrEL-PTX, whereas the clearance (CL) was lower. These results support the expectation that IL-PTX could provide the advantage of slower elimination. It has been reported that drug solutions or suspensions with small particles size (10–50 nm) could be retained in systemic circulation for extended periods as a result of the enhanced permeability and retention (EPR) effect preventing encapsulated material from being taken up and phagocytized by the reticuloendothelial system (RES).³⁴ The biocompatibility of the IL reported (choline as the cation and glycine as the anion) and the small particle size could be the reason for the advantageous PK profile of IL-PTX.

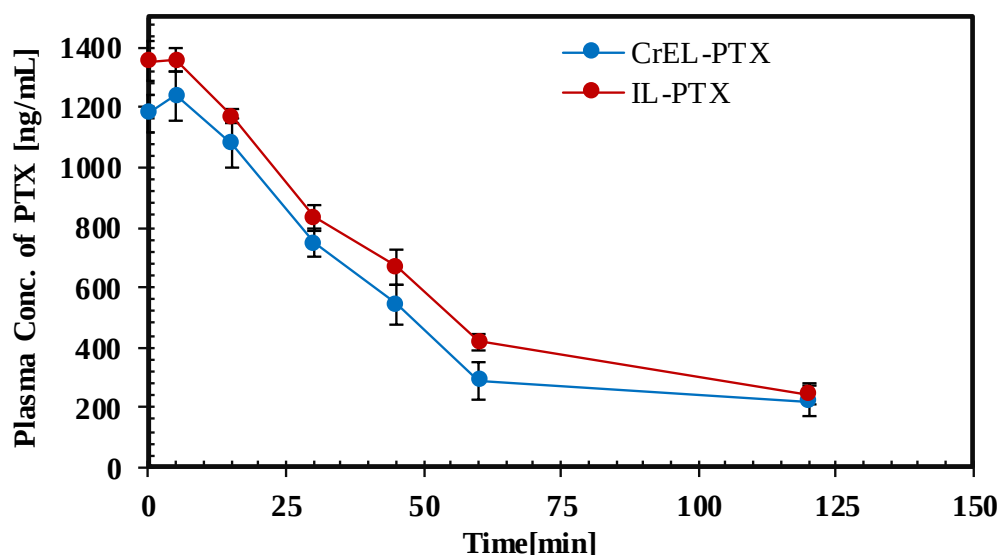


Figure 3-9. The plasma concentration-time profile of PTX after intravenous administration of IL-PTX and CrEL-PTX as a single dose of 2.5 mg/kg of PTX in C57BL/6 mice. Data were calculated as means $\pm$ SD (n = 3).

3.4.6 *In Vitro/Vivo* Antitumor Activity

B16F10 cells and B16F10 tumor-bearing C57BL/6 mice were used to investigate the antitumor activity of the IL-PTX formulation. Before proceeding with an *in vivo* experiment, the *in vitro* cytotoxicity of IL-PTX and CrEL-PTX against B16F10 cells was checked. Fig.3-4 shows the percentage cell viability after 24 h incubation with IL-PTX, IL-[-]PTX, CrEL-PTX, and CrEL-[-]PTX at different concentrations. A significantly different pattern of cytotoxicity was observed for the IL and CrEL formulations compared with the samples without PTX. At high concentration ($>10 \mu\text{g/mL}$ of PTX or 10% formulation in medium), complete inhibition (cell viability under 20%) of cell division was observed for the formulations without PTX, indicating that both IL and CrEL are unsuitable for cytotoxicity assays at high concentrations. However, at low PTX concentration (under $1 \mu\text{g/mL}$ of PTX or 1% formulations), the inhibitory effects of the IL-[-]PTX and CrEL-[-]PTX formulations were significantly lower, indicating that the placebo formulations of IL and CrEL were not as effective as IL-PTX and CrEL-PTX.

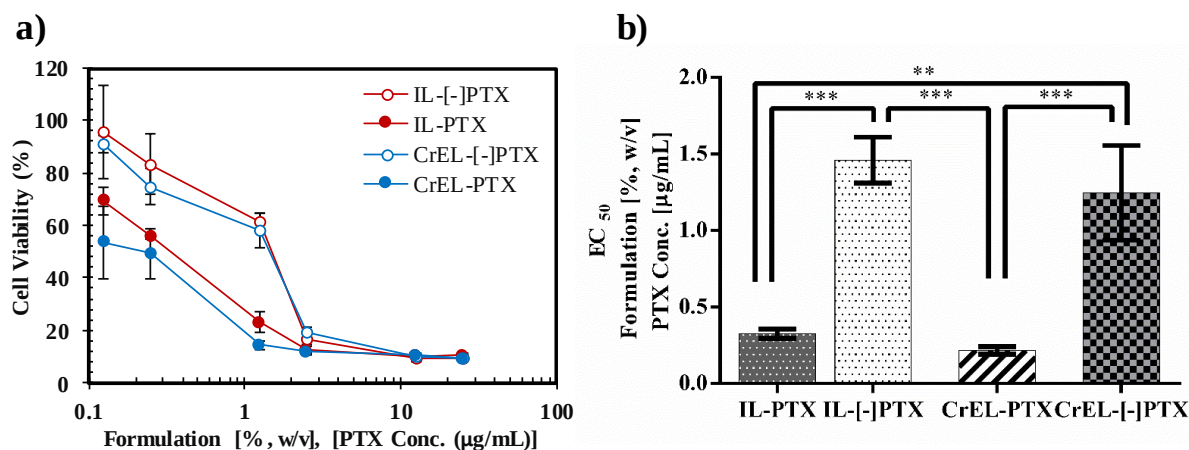


Figure 3-10. (a) Viability of B16F10 cells and (b) EC₅₀ of formulation treated with various concentrations of IL/CrEL with/without PTX. Data were calculated as means $\pm$ SD (n = 3).

** $p < 0.01$, *** $p < 0.001$.

Fig.3-5 (a) shows the *in vivo* antitumor activity of the IL-PTX and CrEL-PTX formulations on B16F10 tumor-bearing C57BL/6 mice following single dose administration. In this experiment, the initial tumor volume was larger than 350 mm³ and no significant difference in tumor size was observed between the control (PBS) and placebo groups. A slight increase in mouse survival time was observed in the case of IL-PTX and CrEL-PTX compared with the control and placebo. The tumor size reached at the developed stage and the PTX concentration used in the formulations (IL and CrEL) were not sufficient to show a significant effect on tumor mass reduction.

In the case of multi-dose administration, a significant difference in the antitumor activity was observed for IL-PTX and CrEL-PTX compared with the control and placebos. As shown in Fig.3-5 (b), both IL-PTX and CrEL-PTX markedly inhibited the growth of B16F10 tumor and tumor size, and increased the mouse survival time. It should be noted that the formulations were administrated at day 14 after B16F10 inoculation when the tumors were > 350 mm³ in volume. Before day 10 post administration, when three doses had been given, the mice showed tumor sizes of ~2500 mm³ for the control and placebo groups and were euthanized. A similar pattern of inhibited tumor growth was observed for both the IL-PTX and CrEL-PTX formulations.

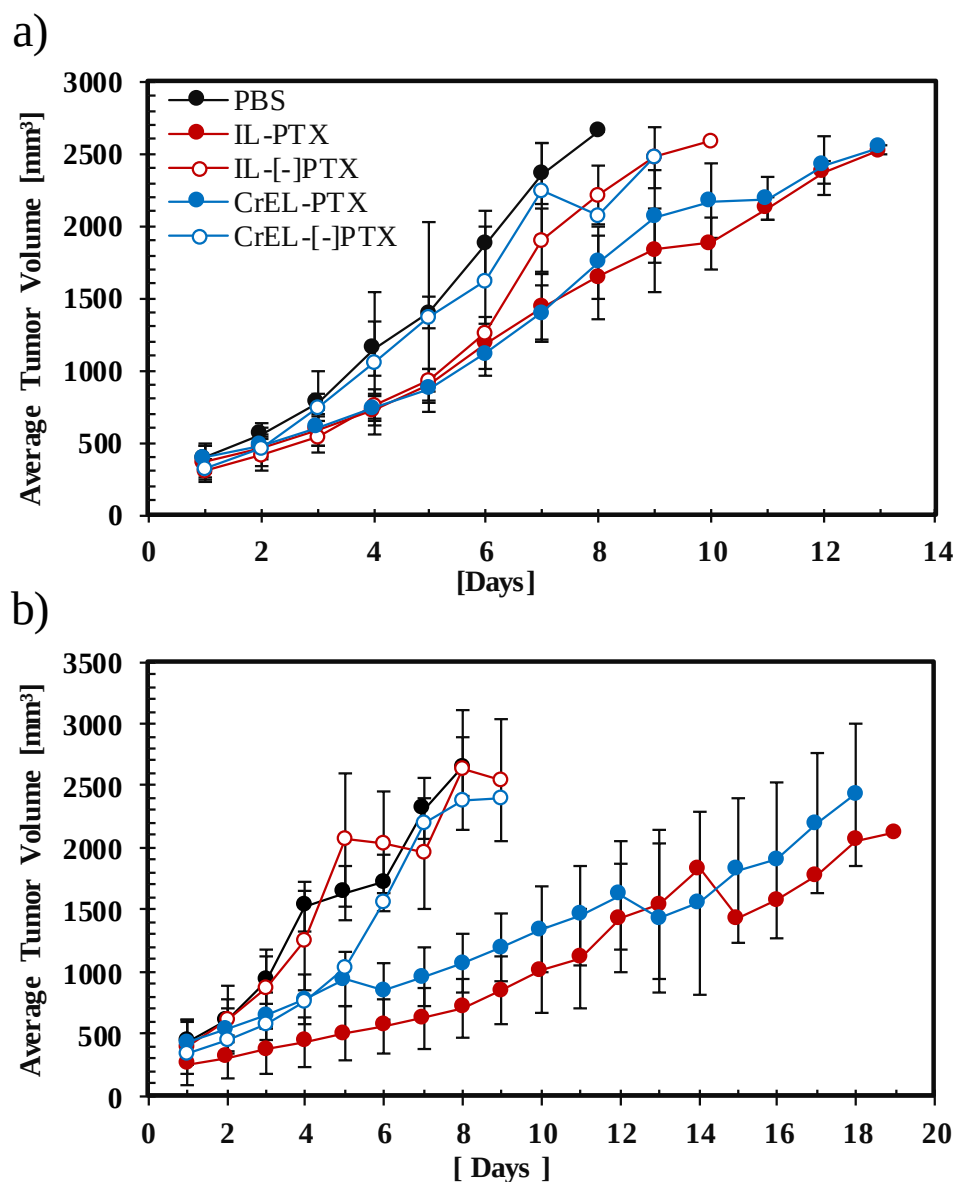


Figure 3-11. Tumor growth inhibition of different formulations of IL and CrEL with and without PTX for (a) single dose and (b) multi-dose. Intratumoral (IT) administration at 2.5 mg/kg of PTX per injection was conducted. Slide calipers were used to measure the tumor size daily. Data were calculated as means $\pm$ SD (n = 5).

Fig.3-6 shows the antitumor activity at different phases of tumor growth for multi-dose administration. The IL-PTX formulation was administrated at three different stages of tumor growth including when the tumor volume was $< 50 \text{ mm}^3$, $< 150 \text{ mm}^3$, and $> 250 \text{ mm}^3$ with five repeat doses each after 2 days. In the early phase, when the mean tumor size was $< 50 \text{ mm}^3$, both the IL and CrEL formulations with PTX completely inhibited the tumor growth and after 5 doses the tumor mass was no longer detected. In the other two cases, slow tumor growth

inhibition was observed in the initial two weeks post administration compared with the control group. Subsequently, tumors regrew faster reaching a size of $\sim 2500 \text{ mm}^3$ and finally mice were euthanized. It was therefore possible to reduce tumor growth and for complete recovery from the tumor to be achieved, when mice were treated with the IL-PTX and CrEL-PTX formulations in the early phase of tumor growth.

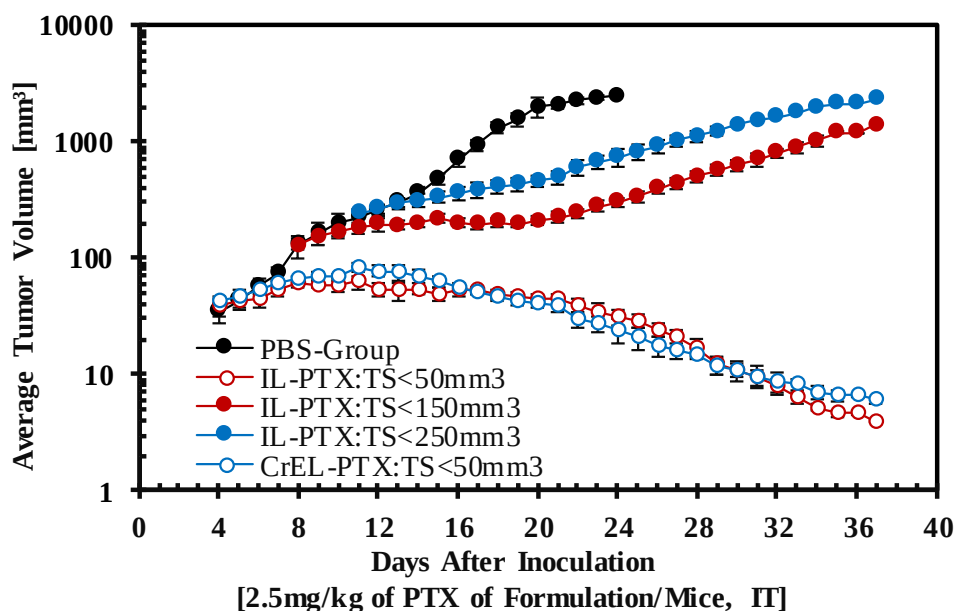


Figure 3-12. Tumor growth inhibition by IL-PTX and CrEL-PTX by multi-dose intratumoral (IT) administration at 2.5 mg/kg of PTX per injection (Every 48–55 h for five doses). Slide calipers were used to measure the tumor size daily.

Data were calculated as means $\pm$ SD (n = 5).

3.4.7 Evaluation of *In Vivo* Hypersensitivity

For the hypersensitivity study, C57BL/6 mice were divided into three groups, control, IL, and CrEL consisting of 5 mice per group. Only the vehicle (PBS) was used in the control group, whereas IL-PTX and CrEL-PTX formulations (Table 3-1) were used in the IL and CrEL groups, respectively. Fig.3-7 shows the calculated lung/body weight ratio (%) for the control, IL, and CrEL groups at 90 min after IV administration. No significant difference was observed between the control and IL groups, whereas the CrEL group shows a slightly increased lung/body weight ratio (%) compared with the control and IL groups. It has been reported that respiratory symptoms such as exudate accumulation and inflammation can occur as a result of hypersensitivity reactions, leading to enlargement of the lung.¹¹

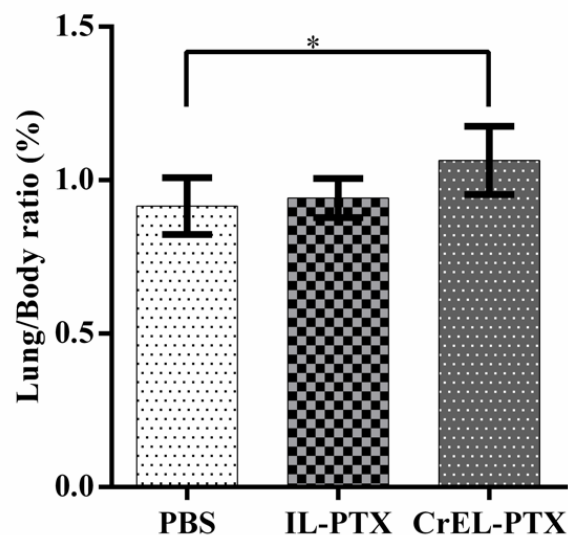


Figure 3-13. Lung/body ratio after intravenous administration of IL-PTX and CrEL-PTX formulations as single doses of 2.5 mg/kg PTX in C57BL/6 mice.

Data were calculated as means $\pm$ SD (n = 5). * p <0.05.

Unsolicited complement activation is one of the major contributory issues in hypersensitivity reactions.³⁵ It has been reported that the CrEL of Taxol is related to the production of the complement component of blood, C3, upon systemic exposure.³⁶ As a result, CrEL may promote the release of several terminal complement components such as C3a and C5a, and these molecules could bind to mast cells to activate histamine release, leading to an increase in microvascular permeability and exudation of inflammatory cells. The alternate phenomenon causes the physiological changes resulting from elevated complement membrane attack complex (SC5b-9) in serum, and accumulation of histamine in serum and lung lesions as a clinical sign of hypersensitivity.³⁶⁻³⁹ Our previous work showed that CrEL-PTX was found to trigger immunological stimulation *in vitro*, which was revealed by the significant increase of the level of cell surface markers (CD54 and CD86) in THP-1 cells.²³ Further confirmation was provided by the *in vivo* experiments using C57BL/6 mice. Sera were collected after 90 min post IV administration of the IL-PTX and CrEL-PTX formulations, the mice were then euthanized, and the lungs were collected and analyzed for SC5b-9 and histamine using the ELISA method as described by the manufacturer. A linear calibration curve was fitted (correlation coefficient, $R^2=0.9979$) for six known standard solutions (Fig.3-S4a). Fig.3-8a shows the estimated SC5b-9 level in serum samples, which indicated an increased SC5b-9 content for CrEL-PTX compared with the control and IL-PTX groups.

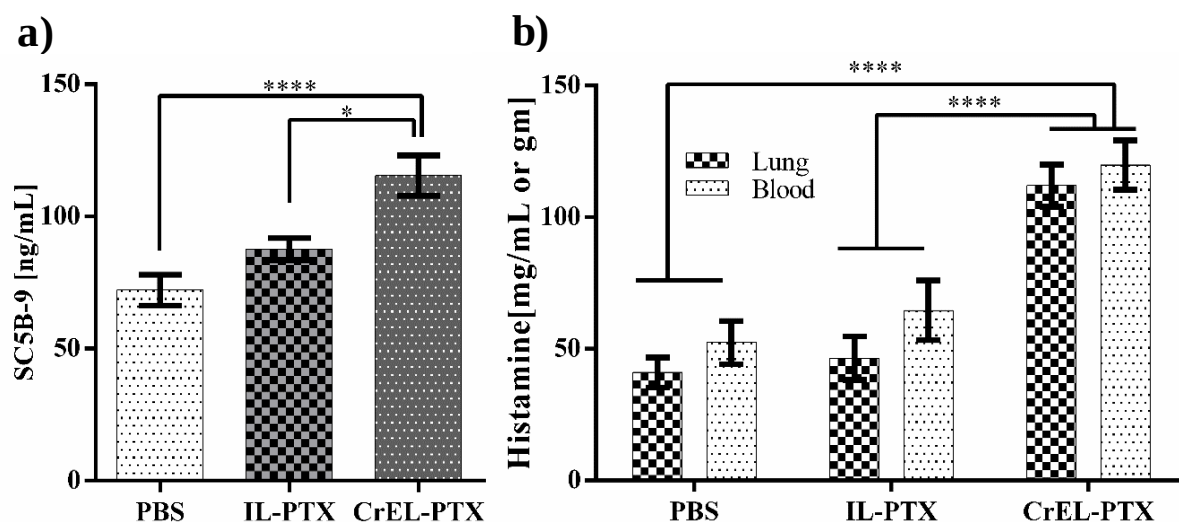


Figure 3-14. The effects of IL-PTX and CrEL-PTX on (a) serum complement activation based on the release of complement split SC5B-9 and (b) serum/lung histamine release for a single dose of 2.5 mg/kg of PTX 90 min after administration in C57BL/6 mice, measured using commercial ELISA kits. Data were calculated as means $\pm$ SD ($n = 5$). * $p < 0.05$, **** $p < 0.0001$.

The SC5b-9 content of the IL-group was slightly higher than the control, however the difference was not found to be significant. For histamine analysis, a logarithmic curve was established (correlation coefficient, $R^2=0.98$) using known standard solutions (Fig.3-S4b & 3-S4c). The histamine content was also found to be significantly higher for CrEL-PTX compared with the control and IL-PTX groups, both in serum and for lung lesions, whereas no significant difference was observed between the control and IL-PTX groups. Moreover, the histamine content of the serum was found to be higher than for lung lesions, although mice were exposed to both the IL-PTX and Taxol formulations for the same time as the control (Fig.3-8b). Complement activation and the resulting synergistic effects were not induced by exposure to the IL-PTX formulation to the extent they were induced by Taxol. This was the first time the IL-based PTX formulation was measured to investigate the complement activation pathway, and the findings were consistent with previous results, such as those for a liposome-based alternative PTX formulation and an ionically charged PTX-lipid complex.⁴⁰⁻⁴² However, in future work, it will be important to elucidate the exact mechanism of action related to complement activation and hypersensitivity reactions induced by IL-based PTX formulations.

3.5 Conclusions

The obtained results indicated that an IL-mediated PTX formulation could be used for therapeutic application. The tailored IL can be used as an alternative material to conventional organic solvent or surfactant for drug delivery systems, and can abate unwanted side effects. The IL or IL-mediated formulations can be administrated intravenously by maintaining physiological buffer conditions (pH and salt concentration). *In vivo* pharmacokinetic data showed that the IL-PTX formulation has a longer systemic circulation time and slower elimination rate than the CrEL-PTX formulation. Furthermore, *in vivo* antitumor and hypersensitivity experiments in C57BL/6 mice showed that the IL-mediated PTX formulation has similar antitumor activity to CrEL-PTX, but a significantly smaller hypersensitivity effect compared with CrEL-PTX. Therefore, the IL-mediated formulation is potentially an effective and safe drug delivery system for PTX.

Supporting Data for Chapter-3

Tables

Table 3-S1. pH and salt's concentrations (mM) of different preparations of IL_{only}[-]PTX for IV injection safety assessment.

		IL in PBS Solution						
IL conc. (% w/v)		0.5	1.0	1.50	2	2.50	5	5
pH of the solution		6.75	6.71	6.69	6.75	6.78	6.82	10.87
Different Salt Conc. [mM]	IL:	28.05	56.11	84.16	112.21	140.27	280.53	280.53
	HCl:	0.19	0.38	0.58	0.77	0.96	1.92	
	NaCl:	138.69	123.29	107.88	92.47	77.06		
	Na ₂ HPO ₄ :	4.59	4.08	3.57	3.06	2.55		
	KH ₂ PO ₄ :	1.39	1.23	1.09	0.92	0.77		
Total Salt Conc. [mM]:		172.91	185.09	197.28	209.43	221.61	282.45	280.53

Table 3-S2. pH and salt's concentrations (mM) of different preparations of IL-[-]PTX for IV injection safety assessment.

		IL/T-80/EtOH as Vehicle		
IL conc. (% w/v)		1.0	1.5	2.0
[mM]		56.11	84.16	112.21
pH of the solution		7.02	6.92	6.89

Table 3-S3. The symptoms and severity of hypersensitivity reactions

Grade	Clinical signs
0/-	Normal
1/+	Disturbance, head shaking
2/++	Shortness of breath, drowsiness
3/+++	Dyspnea, syncope, gatism
4/++++	Mortality

Figures

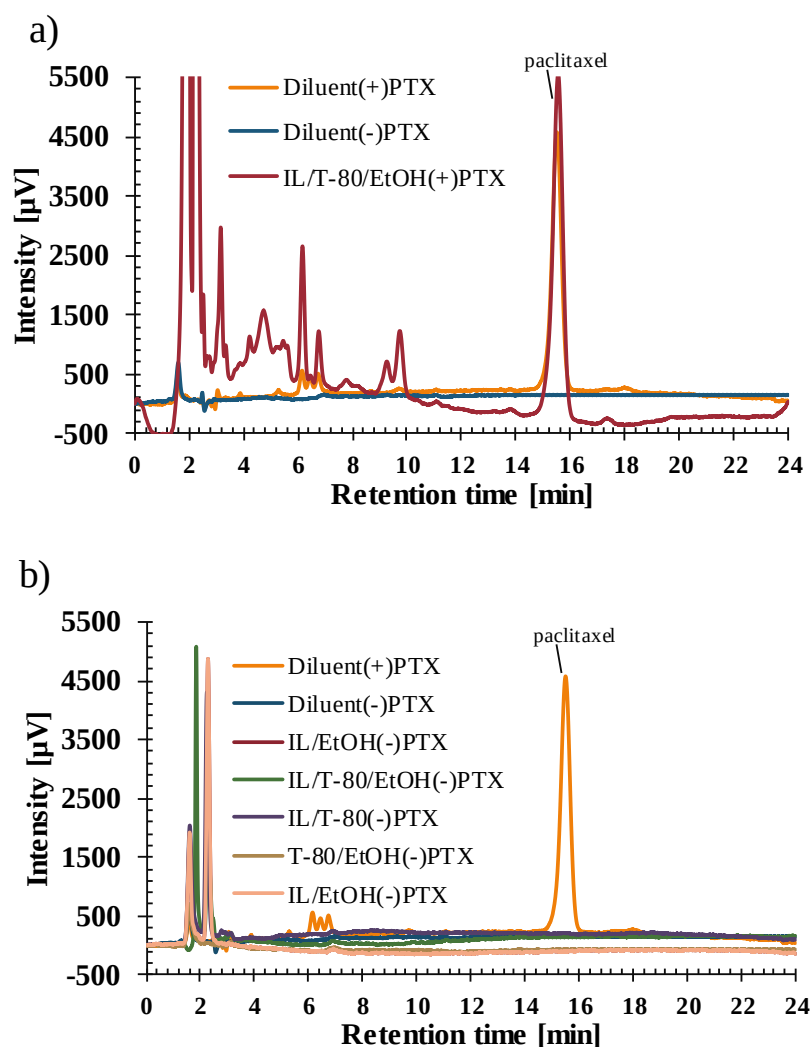


Figure 3-S1. a) Chromatographic separations of PTX during HPLC analysis in standard PTX solution and IL-mediated PTX preparation, b) all possible placebo of IL-mediated preparations. Diluent(+)PTX and IL/T-80/EtOH(+)PTX represent the standard PTX and IL-PTX preparation of 0.5mg/ml of PTX during stability study, respectively.

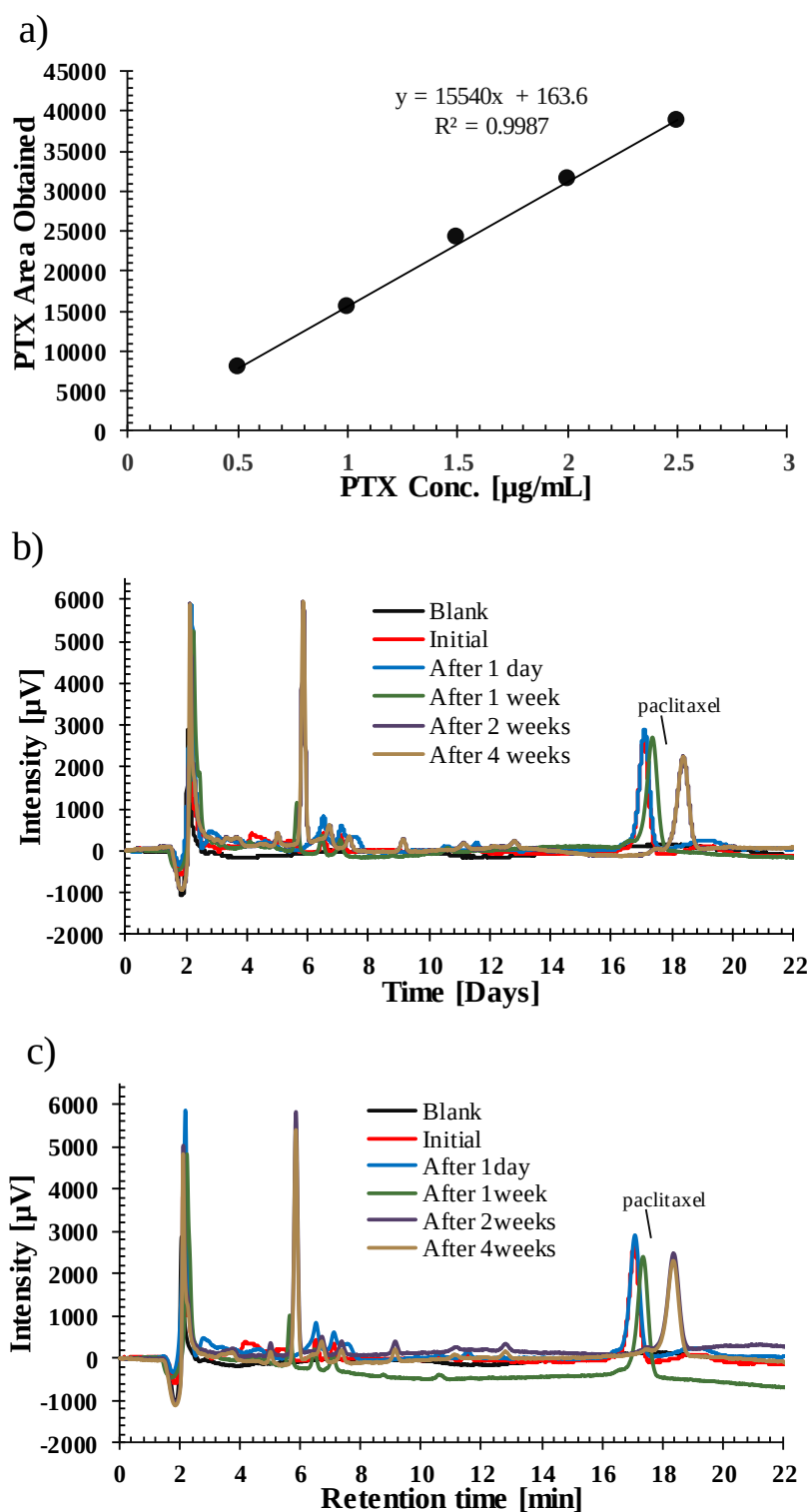


Figure 3-S2. a) Calibration curve prepared by the area obtained from different concentration of Standard (known) PTX solutions during stability study of IL-PTX preparation. Chromatograms of IL-PTX preparations stored in b) refrigeration (2-8°C) c) room temperature condition at different time intervals.

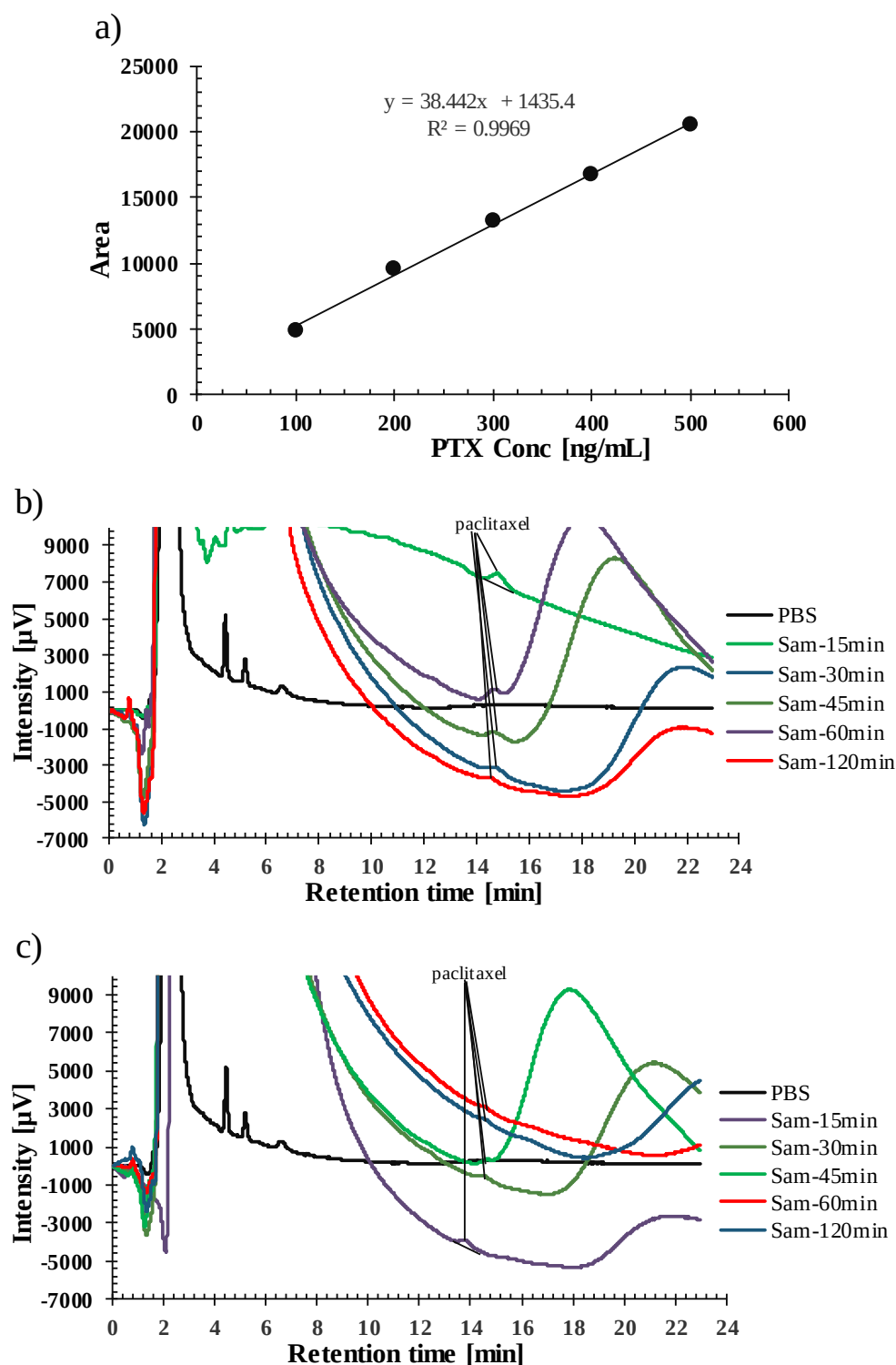


Figure 3-S3. a) Calibration curve prepared by the area obtained from different concentration of standard (known) PTX solutions during pharmacokinetics study. Chromatographs of processed plasma samples obtained after a single dose intravenous administration of IL-PTX(b) & CrEL-PTX(c) preparation at 2.5mg/kg of PTX in C57BL mice during pharmacokinetics study.

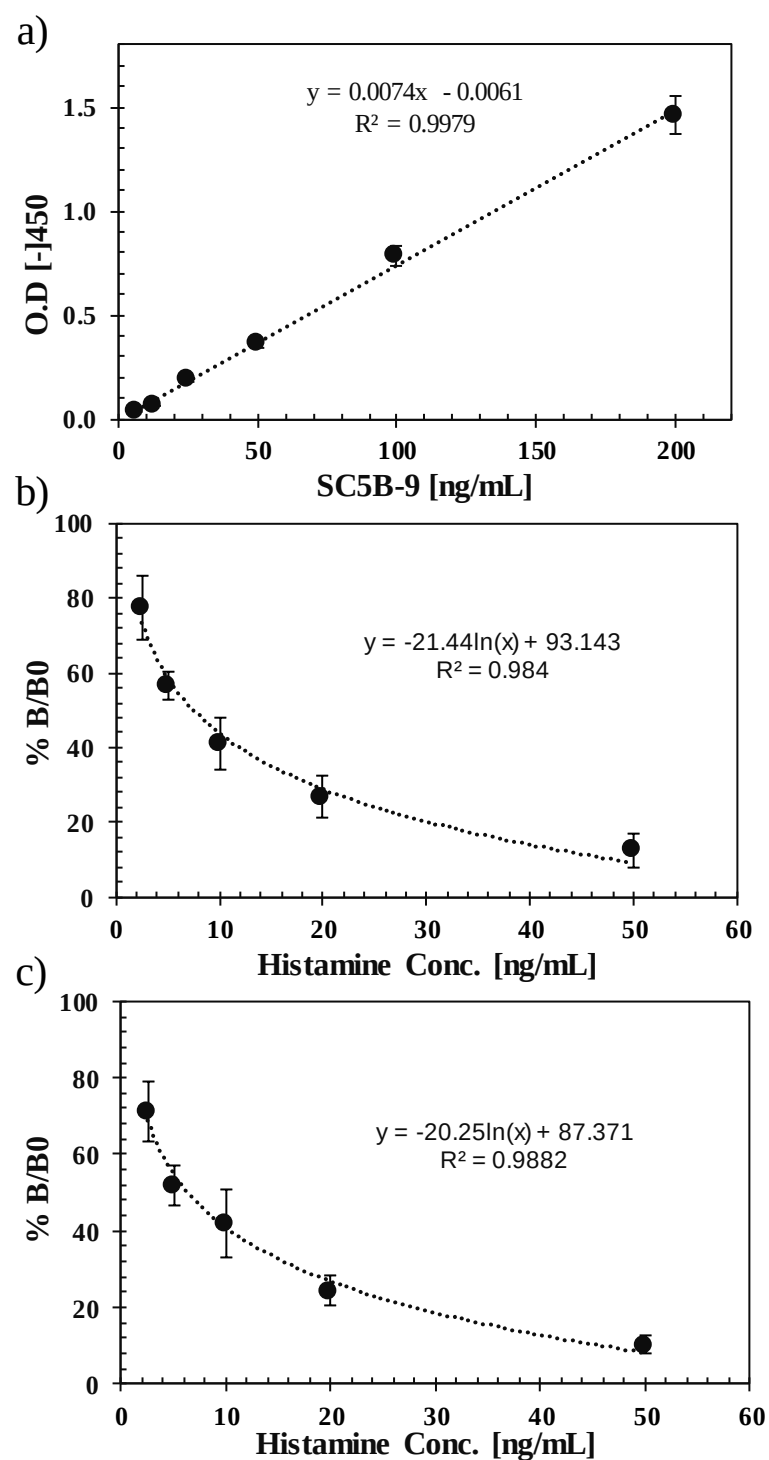


Figure 3-S4. Calibration curves for estimation of a) serum SC5B-9 release in blood samples, histamine release from b) blood samples & c) lung's lesions.

References

- (1) Rowinsky, E. K.; Cazenave, L. A.; Donehower, R. C. Taxol: A Novel Investigational Antimicrotubule Agent. *Journal of the National Cancer Institute*. **1990**.
- (2) Jordan, M. A.; Wilson, L. Microtubules as a Target for Anticancer Drugs. *Nat. Rev. Cancer* **2004**.
- (3) Kavallaris, M. Microtubules and Resistance to Tubulin-Binding Agents. *Nature Reviews Cancer*. 2010.
- (4) Khmelnitsky, Y. L.; Budde, C.; Arnold, J. M.; Usyatinsky, A.; Clark, D. S.; Dordick, J. S. Synthesis of Water-Soluble Paclitaxel Derivatives by Enzymatic Acylation [10]. *J. Am. Chem. Soc.* **1997**, 119 (47), 11554–11555.
- (5) Sparreboom, A.; van Asperen, J.; Mayer, U.; Schinkel, A. H.; Smit, J. W.; Meijer, D. K.; Borst, P.; Nooijen, W. J.; Beijnen, J. H.; van Tellingen, O. Limited Oral Bioavailability and Active Epithelial Excretion of Paclitaxel (Taxol) Caused by P-Glycoprotein in the Intestine. *Proc. Natl. Acad. Sci. U. S. A.* **1997**.
- (6) Singla, A. K.; Garg, A.; Aggarwal, D. Paclitaxel and Its Formulations. *Int. J. Pharm.* **2002**, 235 (1–2), 179–192.
- (7) Gelderblom, H.; Verweij, J.; Nooter, K.; Sparreboom, A. Cremophor EL: The Drawbacks and Advantages of Vehicle Selection for Drug Formulation. *Eur. J. Cancer* **2001**, 37 (13), 1590–1598.
- (8) Dorr, R. T. Pharmacology and Toxicology of Cremophor EL Diluent. *Ann. Pharmacother.* **1994**, 28 (5), S11–S14.
- (9) Szebeni, J.; Muggia, F. M.; Alving, C. R. Complement Activation by Cremophor EL as a Possible Contributor to Hypersensitivity to Paclitaxel: An *in Vitro* Study. *J. Natl. Cancer Inst.* **1998**, 90 (4), 300–306.
- (10) Weiss, R. B.; Donehower, R. C.; Wiernik, P. H.; Ohnuma, T.; Gralla, R. J.; Trump, D. L.; Baker, J. R.; Van Echo, D. A.; Von Hoff, D. D.; Leyland-Jones, B. Hypersensitivity Reactions from Taxol. *J. Clin. Oncol.* **1990**, 8 (7), 1263–1268.
- (11) Wang, H.; Cheng, G.; Du, Y.; Ye, L.; Chen, W.; Zhang, L.; Wang, T.; Tian, J.; Fu, F. Hypersensitivity Reaction Studies of a Polyethoxylated Castor Oil-Free, Liposome-Based Alternative Paclitaxel Formulation. *Mol. Med. Rep.* **2013**, 7 (3), 947–952.
- (12) Fracasso, P. M.; Picus, J.; Wildi, J. D.; Goodner, S. A.; Creekmore, A. N.; Gao, F.; Govindan, R.; Ellis, M. J.; Tan, B. R.; Linette, G. P.; et al. Phase 1 and Pharmacokinetic Study of Weekly Docosahexaenoic Acid-Paclitaxel, Taxoprexin®, in Resistant Solid Tumor Malignancies. *Cancer Chemother. Pharmacol.* **2009**, 63 (3), 451–458.
- (13) Moreno-Aspitia, A.; Perez, E. A. Nanoparticle Albumin-Bound Paclitaxel (ABI-007): A Newer Taxane Alternative in Breast Cancer. *Future Oncol.* **2005**, 1 (6), 755–762.
- (14) Kim, S. Y.; Hwang, J. Y.; Seo, J. W.; Shin, U. S. Production of CNT-Taxol-Embedded PCL Microspheres Using an Ammonium-Based Room Temperature Ionic Liquid: As a Sustained Drug Delivery System. *J. Colloid Interface Sci.* **2015**, 442, 147–153.
- (15) Shamshina, J. L.; Barber, P. S.; Rogers, R. D. Ionic Liquids in Drug Delivery. *Expert*

- Opin. Drug Deliv.* **2013**, *10* (10), 1367–1381.
- (16) Austen Angell, C.; Ansari, Y.; Zhao, Z. Ionic Liquids: Past, Present and Future. *Faraday Discuss.* **2012**, *154* (January), 9–27.
 - (17) Moniruzzaman, M.; Goto, M. Ionic Liquids: Future Solvents and Reagents for Pharmaceuticals. *J. Chem. Eng. Japan* **2011**, *44* (6), 370–381.
 - (18) Adawiyah, N.; Moniruzzaman, M.; Hawatulaila, S.; Goto, M. Ionic Liquids as a Potential Tool for Drug Delivery Systems. *MedChemComm.* The Royal Society of Chemistry October 12, 2016, pp 1881–1897.
 - (19) Matsumura, Y. Preclinical and Clinical Studies of Anticancer Drug-Incorporated Polymeric Micelles. *J. Drug Target.* **2007**, *15* (7–8), 507–517.
 - (20) Gradishar, W. J. Albumin-Bound Paclitaxel: A next-Generation Taxane. *Expert Opin. Pharmacother.* **2006**, *7* (8), 1041–1053.
 - (21) Elsadek, B.; Kratz, F. Impact of Albumin on Drug Delivery — New Applications on the Horizon. *J. Control. Release* **2012**, *157* (1), 4–28.
 - (22) Chowdhury, M. R.; Moshikur, R. M.; Wakabayashi, R.; Tahara, Y.; Kamiya, N.; Moniruzzaman, M.; Goto, M. Ionic-Liquid-Based Paclitaxel Preparation: A New Potential Formulation for Cancer Treatment. *Mol. Pharm.* **2018**, *15* (6), 2484–2488.
 - (23) Chowdhury, M. R.; Moshikur, R. M.; Wakabayashi, R.; Tahara, Y.; Kamiya, N.; Moniruzzaman, M.; Goto, M. Ionic-Liquid-Based Paclitaxel Preparation: A New Potential Formulation for Cancer Treatment. *Mol. Pharm.* **2018**, *15* (6), 2484–2488.
 - (24) Wang, Y.; Wu, K. C.; Zhao, B. X.; Zhao, X.; Wang, X.; Chen, S.; Nie, S. F.; Pan, W. S.; Zhang, X.; Zhang, Q. A Novel Paclitaxel Microemulsion Containing a Reduced Amount of Cremophor EL: Pharmacokinetics, Biodistribution, and In Vivo Antitumor Efficacy and Safety. *J. Biomed. Biotechnol.* **2011**, *2011*, 10.
 - (25) Sznitowska, M.; Klunder, M.; Placzek, M. Paclitaxel Solubility in Aqueous Dispersions and Mixed Micellar Solutions of Lecithin. *Chem. Pharm. Bull. (Tokyo)*. **2008**, *56* (1), 70–74.
 - (26) Moniruzzaman, M.; Tamura, M.; Tahara, Y.; Kamiya, N.; Goto, M. Ionic Liquid-in-Oil Microemulsion as a Potential Carrier of Sparingly Soluble Drug: Characterization and Cytotoxicity Evaluation. *Int. J. Pharm.* **2010**, *400* (1–2), 243–250.
 - (27) Moniruzzaman, M.; Mahmood, H.; Goto, M. Ionic Liquid Based Nanocarriers for Topical and Transdermal Drug Delivery. In *RSC Smart Materials*; Royal Society of Chemistry, 2018; Vol. 2018-Janua, pp 390–403.
 - (28) Araki, S.; Wakabayashi, R.; Moniruzzaman, M.; Kamiya, N.; Goto, M. Ionic Liquid-Mediated Transcutaneous Protein Delivery with Solid-in-Oil Nanodispersions. *Med. Chem. Commun.* **2015**, *6* (12), 2124–2128.
 - (29) Hou, X.-D. D.; Liu, Q.-P. P.; Smith, T. J.; Li, N.; Zong, M.-H. H. Evaluation of Toxicity and Biodegradability of Cholinium Amino Acids Ionic Liquids. *PLoS One* **2013**, *8* (3), e59145.
 - (30) De Santis, S.; Masci, G.; Casciotta, F.; Caminiti, R.; Scarpellini, E.; Campetella, M.; Gontrani, L. Cholinium-Amino Acid Based Ionic Liquids: A New Method of Synthesis

- and Physico-Chemical Characterization. *Phys. Chem. Chem. Phys.* **2015**, *17* (32), 20687–20698.
- (31) Times, P.; Pramanick, S.; Pharmaceuticals, E.; Chandel, V.; Pharma, A.; Pramanick, S. Excipient Selection In Parenteral Formulation Development Article Excipient Selection In Parenteral Formulation Development. **2016**, *45* (March 2013), 65–77.
 - (32) Strickley, R. G. Solubilizing Excipients in Oral and Injectable Formulations. *Pharm. Res.* **2004**, *21* (2), 201–230.
 - (33) Moniruzzaman, M.; Kamiya, N.; Goto, M. Ionic Liquid Based Microemulsion with Pharmaceutically Accepted Components: Formulation and Potential Applications. *J. Colloid Interface Sci.* **2010**, *352* (1), 136–142.
 - (34) He, L.; Wang, G. L.; Zhang, Q. An Alternative Paclitaxel Microemulsion Formulation: Hypersensitivity Evaluation and Pharmacokinetic Profile. *Int. J. Pharm.* **2003**, *250* (1), 45–50.
 - (35) Weiszhar, Z.; Czucz, J.; Révész, C.; Rosivall, L.; Szebeni, J.; Rozsnyay, Z. Complement Activation by Polyethoxylated Pharmaceutical Surfactants: Cremophor-EL, Tween-80 and Tween-20. *Eur. J. Pharm. Sci.* **2012**, *45* (4), 492–498.
 - (36) Weiszhar, Z.; Czucz, J.; Révész, C.; Rosivall, L.; Szebeni, J.; Rozsnyay, Z. Complement Activation by Polyethoxylated Pharmaceutical Surfactants: Cremophor-EL, Tween-80 and Tween-20. *Eur. J. Pharm. Sci.* **2012**, *45* (4), 492–498.
 - (37) Ajuebor, M. N.; Das, A. M.; Virág, L.; Flower, R. J.; Szabó, C.; Perretti, M.; Regoli, D.; Tedesco, F. Role of Resident Peritoneal Macrophages and Mast Cells in Chemokine Production and Neutrophil Migration in Acute Inflammation: Evidence for an Inhibitory Loop Involving Endogenous IL-10. *J. Immunol.* **1999**, *162* (3), 1685–1691.
 - (38) Karasuyama, H.; Tsujimura, Y.; Obata, K.; Mukai, K. Role for Basophils in Systemic Anaphylaxis. In *Anaphylaxis*; KARGER: Basel, 2010; Vol. 95, pp 85–97.
 - (39) Szebeni, J. Complement Activation-Related Pseudoallergy: A New Class of Drug-Induced Acute Immune Toxicity. *Toxicology* **2005**, *216* (2–3), 106–121.
 - (40) Cabanes, A.; Briggs, K. E.; Gokhale, P. C.; Treat, J. A.; Rahman, A. Comparative in Vivo Studies with Paclitaxel and Liposome-Encapsulated Paclitaxel. *Int. J. Oncol.* **1998**, *12* (5), 1035–1040.
 - (41) Xia, X.-J.; Guo, R.-F.; Liu, Y.-L.; Zhang, P.-X.; Zhou, C.-P.; Jin, D.-J.; Wang, R.-Y. Formulation, Characterization and Hypersensitivity Evaluation of an Intravenous Emulsion Loaded with a Paclitaxel-Cholesterol Complex. *Chem. Pharm. Bull. (Tokyo)*. **2011**, *59* (3), 321–326.
 - (42) Fasol, U.; Frost, A.; Büchert, M.; Arends, J.; Fiedler, U.; Scharf, D.; Scheuenpflug, J.; Mross, K. Vascular and Pharmacokinetic Effects of EndoTAG-1 in Patients with Advanced Cancer and Liver Metastasis. *Ann. Oncol.* **2012**, *23* (4), 1030–1036.

Nomenclature and Abbreviations for Chapter-3

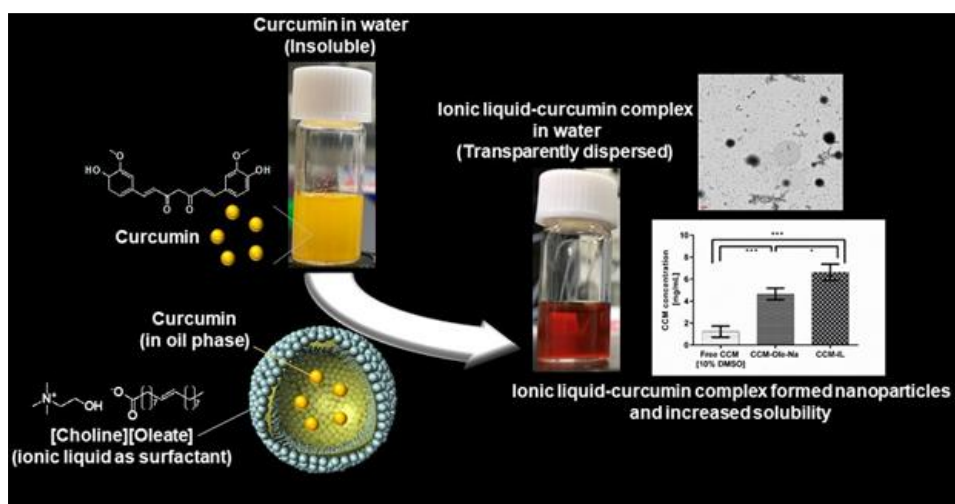
Abbreviation	Name/description
AUC	Area under curve
CrEL	Cremophor EL (Polyethoxylated castor oil)
C ₀	Concentration at time zero.
C _{max}	Maximum concentration
CL	Clearance
EtOH	Ethanol, anhydrous
IL	Ionic Liquid
PK	Pharmacokinetics
PTX	Paclitaxel
T-80	Tween-80 (Polyoxyethylene 20 sorbitan monooleate)
t _{1/2}	Elimination half life
V _d	Volume of distribution

CHAPTER-4

DEVELOPMENT OF A NOVEL IONIC LIQUID-CURCUMIN COMPLEX TO ENHANCE ITS SOLUBILITY, STABILITY, AND ACTIVITY

4.1 Abstract

Curcumin (CCM), a natural polyphenol, has attracted significant attention as a potent anticancer agent and antioxidant. However, the insolubility of CCM in water restricts its therapeutic applications. To overcome this limitation, this study reports the encapsulation of CCM by the ionic liquid (IL) choline oleic acid, using a one-step emulsification and rapid freeze-drying process. The developed CCM-IL complex could be readily dispersed in water with a significantly enhanced solubility of ~8 mg/mL and half-life ($t_{1/2}$) of ~260 min compared with free CCM (solubility ~30 nM and $t_{1/2}$ ~20 min). Comparison with a similar complex encapsulated using oleic acid sodium salt (Ole-Na), led to the conclusion that both the cation and anion of the IL have significant contributory roles in the solubilization and stabilization of CCM in the complex. Furthermore, release studies in three different physiological fluids showed bi-phasic profiles with an initial burst release of CCM over a few hours, followed by slow sustained release up to 24 h. Finally, the anticancer activity of the CCM-IL complex was found to be significantly higher when used to treat cancer cells (HepG2) compared with normal cells (fibroblast). The emulsification and freeze-drying process using an IL consisting of a long chain carbon backbone, as a surfactant, may provide an alternative way of enhancing the solubility of poorly water-soluble compounds.



Graphical Presentation of IL-CCM Complex

4.2 Introduction

Curcumin (CCM), a naturally occurring polyphenol derived from the turmeric plant, has been receiving significant attention over recent decades because of its diverse biological activities, including anticancer, antioxidant, anti-amyloid, anti-inflammatory, antidiabetic, antibiotic, and antiviral activities.¹ CCM is well recognized as a promising drug candidate for the treatment of a large number of diseases such as cancer,^{2–5} neurodegenerative diseases,⁶ infectious diseases,⁷ and diabetes.⁸ However, the application of CCM in therapeutic treatments has been hindered by three major limitations: its low aqueous solubility (~30 nM in H₂O), its chemical instability in aqueous solution under physiological pH 7.4 (half-life, $t_{1/2}$ ~20 min), and its low cellular uptake.⁵ It has been reported that CCM tends to insert deeply into the cell membrane through hydrophobic interaction with the fatty acyl chains and hydrogen bonding with the phosphate groups of the lipids, in a manner similar to cholesterol. This results in limited amounts of CCM diffusion into the cytoplasm, which is the main target action site of CCM for most of its bioactivities.^{9–11} Generally, organic solvents such as dimethyl sulfoxide (DMSO) and acetone-methanol are used to dissolve CCM; however, these solvents are not safe for biological applications, particularly drug delivery systems.¹² Overcoming these challenges is therefore key to achieving the clinical application of CCM.

To date, the reported attempts to overcome the limitations of CCM include numerous physico-chemical¹³ modifications, such as preparing polymer-conjugates,^{3,14} liposomes,¹⁴ polymeric micelles,¹⁵ silica nanoparticles,¹⁶ supramolecular assemblies,⁵ lipid nano-carriers,¹⁷ and genipin-crosslinked nanoparticles of caseinate and chitosan.¹⁸ However, none of these approaches have been approved for clinical therapeutic application. Moreover, the vehicles (polymers/surfactants used to fabricate the CCM systems) play an inert role during drug delivery. Therefore, designing a vehicle system with biological functions similar/related to those of the active drug molecule could enhance the potential of CCM.

In this study, we propose a one-step emulsification and freeze-drying process for the encapsulation of CCM, using an ionic liquid (IL), [Ch][Ole], consisting of choline as the cation and the long chain fatty acid (oleic acid, C18:1) as the anion. ILs, composed entirely of ions, exist as fluids around or below 100°C¹⁹ and are known as designer solvents because their properties can be tailored to the target application simply by altering the cation/anion.^{20,21,22} For this work, the [Ch][Ole] IL was selected owing to its biocompatibility.^{23,24} Both the cation (choline) and anion (oleic acid) of the IL are widely accepted as biocompatible.^{23,25,24}

Furthermore, oleic acid has been reported to have many interesting biological functions including anti-inflammatory,²⁶ anti-carcinogenic,²⁷ anti-oxidative,²⁸ and immune system activation properties.²⁹ Therefore, combining the therapeutic effects of the surfactant and drug could be a novel approach to the preparation of surfactant-drug complexes. Here, the IL plays the role of a surfactant dissolved in the water phase, and the CCM in the oil phase was encapsulated by the IL to form the CCM-IL complex. The surfactant properties of ILs consist of long-chain carboxylate as anion have been reported in many studies.³⁰ In this study, an oil-in-water emulsion were prepared using the IL in water phase and CCM in oil phase, followed by freeze-drying to form a CCM-IL complex. For comparison, a similar complex was prepared using oleic acid sodium salt (Ole-Na) in the water phase. No specific interactions are required between the hydrophobic drug molecules and the surfactants for these complexes, because the emulsification and freeze-drying processes drive the inclusion of drug in the IL-derived surfactant molecule layer. We developed a CCM-IL complex that can be readily dispersed in water and shows enhanced drug activity and chemical stability.

4.3 Experimental

4.3.1 Materials and Methods

4.3.2 Chemicals

Curcumin (CCM), HPLC grade methanol, acetonitrile, cyclohexane, dimethyl sulfoxide (DMSO) and silver oxide was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Choline Chloride, Oleic Acid and Oleic Acid Sodium Salt (Ole-Na) were procured from Chameleon Guaranteed Reagent, Japan. The choline oleic acid ionic liquid ([Ch][Ole] IL) used in this study was synthesized following the procedure of a previously reported method.³¹ A cell counting kit, WST-8, and Hoechst 4432 solution were purchased from Dojindo Laboratories, Inc. (Kumamoto, Japan). Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin, streptomycin, OPTI-MEM were purchased from Gibco, Life Technologies Corp. (USA). Trypsin/1, 2-ethylenediaminetetraacetic (EDTA) and Dulbecco's phosphate buffered saline (D-PBS) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Milli-Q purification system from Millipore (Molsheim, France) used to prepare de-ionized and ultrapure water throughout the experiment. All other chemicals were analytical grade.

4.3.3 HepG2 and Fibroblast Cell Lines

The human liver carcinoma cell (HepG2 cell) and normal human cell (fibroblast) were procured from RIKEN cell bank (Tsukuba, Japan). HepG2 and fibroblast cells were cultured in EMEM medium and MEM medium, respectively, supplemented with 10 % FBS and 0.1% of antibiotic-antimitotic.

4.3.4 Chromatographic Conditions

The concentrations of CCM from different samples (stability and release study) were estimated by using a HPLC-system consisting of a pump (PU-4180), a photo diode array (PDA) detector (MD-4010), an auto sampler (AS-4050) and a column oven controller (CO-4060) with a interface box (LC-NetII/ADC) and a bottle stand (BS-4000-1) (JASCO International Co. Ltd., Hachioji, Tokyo 193-0835 Japan). The mobile phase was consisted of acetonitrile and Milli-Q water at a ratio of 60:40 (v/v) and delivered at a flow rate of 1.0 mL/min. The chromatographic separation was performed on an Inertsil ODS-3 column (4.6×250 mm, 5 µm, GL Sciences Inc., Shinjuku-ku, Tokyo, 163-1130, Japan) at the wavelength of 445 nm. A linear calibration curves was established within the range from 1.0 µg/mL to 5.0 µg/mL (correlation coefficient, $R^2 = 0.9998$) for analysis of CCM in different sample of stability and release study.

4.3.5 Preparation of CCM-Ole-Na and CCM-IL Complexes

CCM was dissolved in acetone at a concentration of 20 mg/mL. Then 50 µL of the prepared solution was mixed with 100 µL of methanol. To this solution, 0.85 mL of cyclohexane was added to obtain an oil phase. A volume of 4 mL aqueous phase containing either Ole-Na or [Ch][Ole] IL solution (1 mg/mL in Milli-Q water) was added to the oil phase. An oil-in-water (o/w) emulsion was developed in the presence of aqueous phase using a Polytron homogenizer (Kinematica, Bohemia, NY, USA) at 16,000 rpm for 2 min that was immediately frozen in liquid nitrogen for 25-30 min. Then, it was freeze-dried to form a solid CCM-Ole-Na or CCM-IL complexes.

4.3.6 Characterizations of CCM-Ole-Na and CCM-IL Complexes

At first, the hydrodynamic size, polydispersity index (PDI), and zeta potential of CCM-Ole-Na and CCM-IL complexes were measured by dynamic light scattering (DLS: Zetasizer Nano ZS, Malvern Instruments Ltd., USA). Briefly, CCM-Ole-Na and CCM-IL complexes were dispersed in 1 mL of Milli-Q water at concentration of 1 mg/mL of CCM. Then, 0.2 mL of dispersed solution was further diluted to 1 mL by Milli-Q water. The resultant dispersion was used for DLS measurement. Transmission electron micrograph (TEM) analysis was performed as follows: 2 μ L of the resultant dispersion was placed on a copper TEM grid supported by carbon film, incubated for 2 min, and additional solution was absorbed by a filter paper. Then, 2 % uranyl acetate solution in water was used for staining the deposited complexes. Finally, the samples were imaged on a TEM-2010 (JEOL, Tokyo, Japan), operated at an acceleration voltage of 120 kV. The encapsulation efficiency (EE) of CCM by Ole-Na or [Ch][Ole] IL were determined by a filtration method. Briefly, the freeze dried CCM-Ole-Na or CCM-IL complexes were dispersed in Milli-Q water. The suspension was filtrated by using a membrane filter (0.2 μ m). The unloaded CCM should be removed by the filtration process. Finally, samples from un-filtrated and filtrated portion were injected in HPLC system for analysis with appropriate dilution with mobile phase. The EE (%) and Yield (%) were calculated as follows:

$$EE (\%) = \frac{\text{Content of CCM in filtraed portion}}{\text{Content of CCM in unfiltarted portion}} \times 100\%.$$

$$Yield (\%) = \frac{\text{Total CCM entraped in final product}}{\text{Total CCM added at initial}} \times 100\%.$$

4.3.7 IV Solubility of CCM-Ole-Na and CCM-IL Complexes in Milli-Q

The solubility study was performed by adding an excessive amount of CCM-Ole-Na and CCM-IL complexes in a glass vial containing 1.0 mL of Milli-Q water. As a control, excessive amount of free CCM was added in 10 % DMSO in Milli-Q. After addition, the suspension were prepared by shaking and rotating gently the vial for 2 min to obtain a dispersed solution. The undissolved CCM remained as precipitates, which were removed by high centrifugation (15,000 rpm, 5 min). The amount of solubilized drug in supernatant was diluted with mobile phase, mixed well by sonication, filtrated through a 0.22 μ m filter (Milliopore Millex-LG, PTFE, Fisher Scientific, USA) and analyzed using a HPLC-system (JASCO International Co. Ltd., Hachioji, Tokyo, Japan) at 445 nm. Following a literature validation method with slight

modification, the supernatants were diluted with acetonitrile at 50:50 ratio and content of CCM were estimated using a calibration curve prepared by reference standards.

4.3.8 Stability Study of CCM-Ole-Na and CCM-IL Complexes

To perform the stability study, CCM-Ole-Na and CCM-IL complexes containing 0.5 mg/mL of CCM were dissolved in Milli-Q water. Similarly, 0.5 mg/mL of CCM was dispersed in 10 % DMSO in Milli-Q as a control. The dispersions were observed visually to check whether formation of precipitations or clear solution. For the chemical stability, content of CCM was quantitatively analyzed at different time intervals. Briefly, after 0, 10, 20, 30, 60, 90, 120, 180, and 240 min of initial dispersion, the solution was withdrawn, diluted by acetonitrile at a 50:50 ratio, mixed well by vortex, filtrated through a 0.22 μ m filter and analyzed using the HPLC system as described above. The experiment was carried out at room temperature. The content of CCM in each time point was calculated using the calibration curve derived from reference standard. Finally, a graph of concentration (mg/mL) vs time (min) was developed to calculate the half-life ($t_{1/2}$) of CCM using the following equation:

$$\text{Half-life, } t_{1/2} = \frac{0.693}{k}, \text{ where, rate constant, } k = -2.303 \times \text{slope}$$

3.3.9 In-Vitro Release Profile of CCM from CCM-Ole-Na and CCM-IL Complexes

At first, a 96-well plate (CELLSTAR, Greiner Bio-One gmnH, Germany) containing 5,000 of cells was incubated overnight at 37 °C and 5 % CO₂ in an incubator (Espec Corp., BNS-110, Japan). After that, the medium was withdrawn and the CCM-Ole-Na and CCM-IL complexes dispersed in 100 μ L of Opti-MEM at different concentration ranging from 1-25 μ g/mL were added into the wells. Free CCM solution in Opti-MEM, in which DMSO was added to dissolved free CCM that was kept < 0.5 mg/mL in final solution, at the same concentrations as a control. The incubation was performed in triplicates for each sample including the control. After 24 hour incubation, the cells were washed twice with D-PBS and again diluted by WST-8 solution and incubated for 3 h at 37 °C. Finally, the absorbance at 450 nm was measured using a microplate reader (iMark, Bio-Rad, USA). Cell viability was calculated as the percentage of exposed cells that remained viable over controls as follows:

$$\text{Cell Viability (\%)} = (A_{\text{exposed}} / A_{\text{control}}) \times 100 \%$$

Where, A_{exposed} and A_{control} represent the absorbance of exposed and control cells, respectively. The data were collected from at least three independent experiments and presented as mean with the standard deviation.

3.4 Results and Discussion

The very low aqueous solubility and chemical instability are the main barriers to use of CCM in therapeutic applications.^{5,32} Furthermore, CCM is only soluble in a small number of organic solvents, which are not biocompatible. The CCM-IL nano-complexes were obtained using the preparation scheme shown in Fig.4-1.

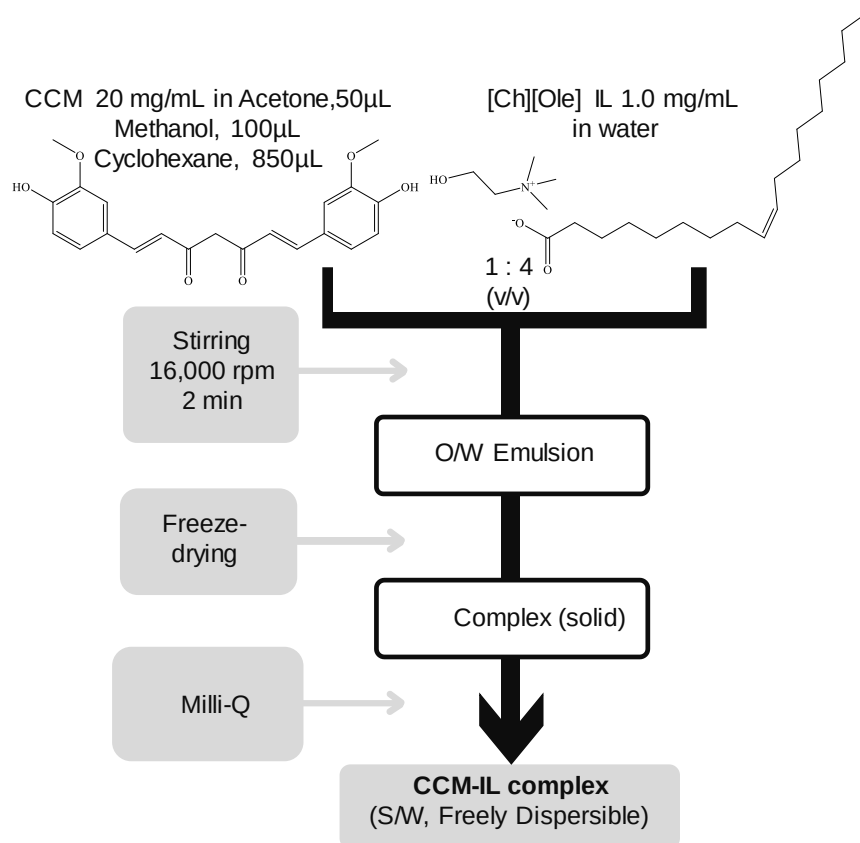


Figure 4-1. Preparation scheme for CCM-IL complexes.

Briefly, an oil phase containing CCM in acetone-methanol-cyclohexane was mixed with a water phase containing [Ch][Ole] IL to form an oil-in-water (o/w) emulsion by homogenizing at 16,000 rpm for 2 min. A solid CCM-IL complex was obtained after freeze-drying the o/w emulsion. A solid CCM complex of oleic acid sodium salt (CCM-Ole-Na) was prepared using the same process to confirm which component of the IL (anion or/and cation) is responsible for the encapsulation process. The obtained solids could be readily dispersed in water to give a precipitation free dispersion (Fig.4-2C and 4-2D). It has been reported that free CCM is poorly soluble in water forming visible clusters or aggregates even in the presence of 10% DMSO (Fig. 4-2A and 4-2B). Oleic acid also separates and is insoluble in water; however, both the IL and Ole-Na are soluble and can act as a surfactant. We therefore conclude that the

emulsification process in the presence of surfactant forces CCM into the oil phase within the water phase, resulting in the coating of CCM with Ole-Na or IL. The anionic long chain carbon backbone (C18:1) of IL or Ole-Na including the cationic part were assumed to be located at the surface surrounded the complex, participating in the important role of inhibiting the random self-aggregation of CCM or the complex in water.

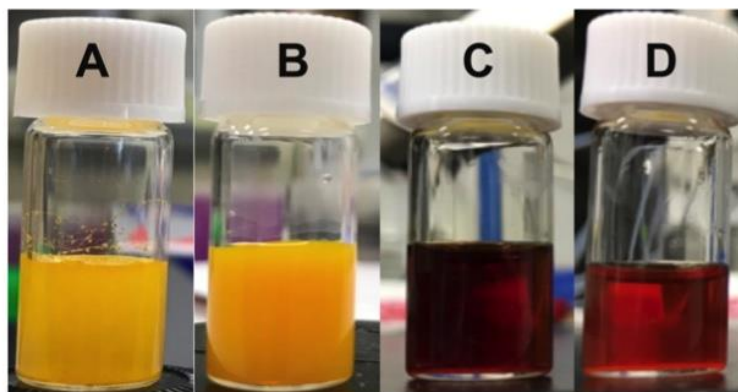


Figure 4-2. Physical appearance of CCM dispersed in water as, A) free CCM, B) free CCM in 10 % DMSO, C) CCM-Ole-Na complex, and D) CCM-IL complex.

Dynamic light scattering (DLS) was used to measure the hydrodynamic size, polydispersity index (PDI), and zeta potential of the complex of a placebo preparation of IL ([$-$]CCM-IL), CCM-Ole-Na, and CCM-IL dispersed in water. As shown in Table 4-1, the mean particle size of the placebo was slightly smaller than that of the CCM-IL complex, indicating the incorporation of CCM into the capsule. Interestingly, compared with the CCM-Ole-Na complex, the CCM-IL complex is significantly larger, possibly owing to the presence of the bulky cholinium group as the cation in the CCM-IL complex. The zeta potentials were found to be similar for all complexes, indicating that the encapsulation process was mainly governed by the long chain carbon backbone, regardless of the form of IL or the Ole-Na. The role of the cation during the encapsulation process may be insignificant, however, this is unclear at this point, and requires further investigation. The complexes were further characterized by transmission electron microscopy (TEM) analysis. It was found that free CCM in water formed a crystalline structure with dimensions around 10–50 μm owing to having very low solubility.³² As shown in Fig.4-3, the nano-complexes of both CCM-Ole-Na and CCM-IL were observed to be spherical in shape, indicating that the oil-in water (o/w) emulsification encapsulation process was efficient for preparing spherical shapes and developing solid-in-water (s/w) nano-

complexes. The Ole-Na or IL component was thought to encapsulate CCM and exhibit it on the surface of the nano-complexes to give the spherical shape shown by TEM analysis, and is in agreement with the DLS data. A filtration method was used to determine the encapsulation efficiency (EE) and yield, which were calculated to be more than 84 % and 89 %, respectively, for both the CCM-Ole-Na and CCM-IL complexes (Table 4-S1).

Table 4-2. Z-average size, PDI, and surface charge of the CCM-complexes dispersed in Milli-Q water. Data are shown as mean $\pm$ standard deviation, n=3. ^aPlacebo that was prepared using a similar procedure as for the CCM-IL complex, but without CCM.

Complex	Particle Size [nm]	PDI	Zeta potential [mV]
^a (-)CCM-IL	285.8 $\pm$ 19.9	0.32 $\pm$ 0.11	-80.7 $\pm$ 5.2
CCM-Ole-Na	155.4 $\pm$ 22.6	0.39 $\pm$ 0.01	-83.9 $\pm$ 7.8
CCM- IL	326.2 $\pm$ 24.4	0.26 $\pm$ 0.02	-84.1 $\pm$ 9.7

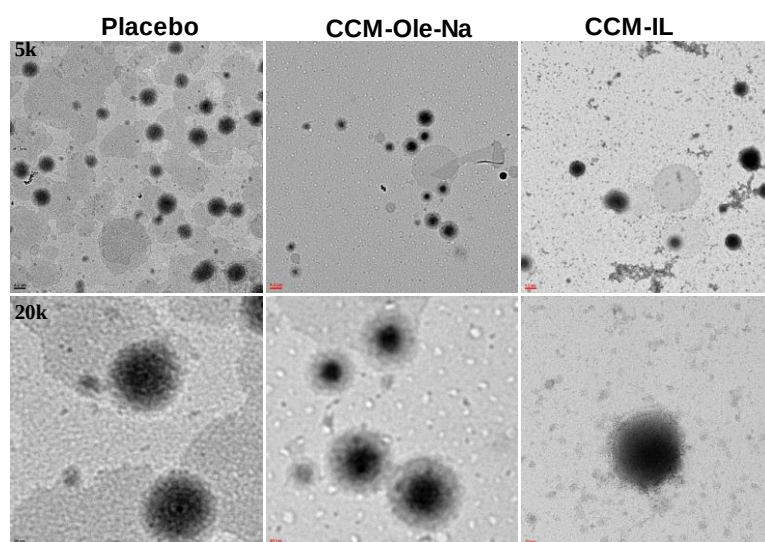


Figure 4-3. TEM images of the (-)CCM-IL (Placebo) (left), CCM-Ole-Na (middle), and CCM-IL (right) complexes. Scale bar: 200 nm (top images, 5K resolution) and 50 nm (bottom images, 20K resolution).

The maximum solubility of CCM in the CCM-Ole-Na and CCM-IL complexes were assessed by measuring the concentration of CCM in supernatant after adding excess solid in water. An equal concentration of CCM in 10 % DMSO was also measured as a control. Free CCM is poorly soluble in water—in the nanomolar (nM) range—while the concentration required to achieve biological activity is in the micromolar (μM) range. A maximum solubility in water greater than millimolar (mM) was calculated for both complexes (Fig.4-4 and Table 4-S1). However, the chemical stability of CCM encapsulated in the complexes analysed by HPLC, revealed that CCM was more stable in CCM-IL complex than CCM-Ole-Na complex. The calculated half-life ($t_{1/2}$) of CCM was found to be significantly enhanced, ~ 4.3 h and 1.3 h in the CCM-IL and CCM-Ole-Na complexes, respectively, compared with free CCM in water (see Table 4-S1 and Fig.4-S1). After initial dispersion in water or 10 % DMSO, free CCM is rapidly degraded, $t_{1/2} \sim 20$ min.⁵ It has been reported that in the presence of water, an autoxidation transformation of CCM starts when incorporation of oxygen forms a hydroperoxide intermediate, bicyclopentadione, causing the Hock cleavage and direct transformation of spiroepoxide to bicyclopentadione by intermolecular hydrolysis of the epoxide.¹¹ Here, the unsaturated long-chain carbon backbone of oleic acid in Ole-Na or [Ch][Ole] IL was thought to participate in the encapsulation process by covering the CCM nanocrystals through hydrogen bonding, van der Waals forces, or π – π interactions. The encapsulation process was primarily governed by the inclusion of CCM within the carbon backbone assembly driven by the emulsification and freeze-drying processes. Owing to the difference of complex sizes, the cationic part of the surfactant was thought to play an important role in complex formation by exhibiting a polar interaction with the nano-crystals. The stability was found to be significantly enhanced in both complexes (Ole-Na and IL) in water, compared with free CCM in water $\pm$ 10 % DMSO (see Fig.4-S1). Furthermore, the CCM-IL showed higher solubility and CCM stability compared with Ole-Na (see Fig.4-4, Fig.4-S1 and Table 4-S1). It is believed that encapsulation causes the formation of a layer consisting of alkyl chains, protecting CCM from autoxidation in the presence of water, and the choline–oleic acid based layer is suitable because of its biocompatibility.^{17,23,25}

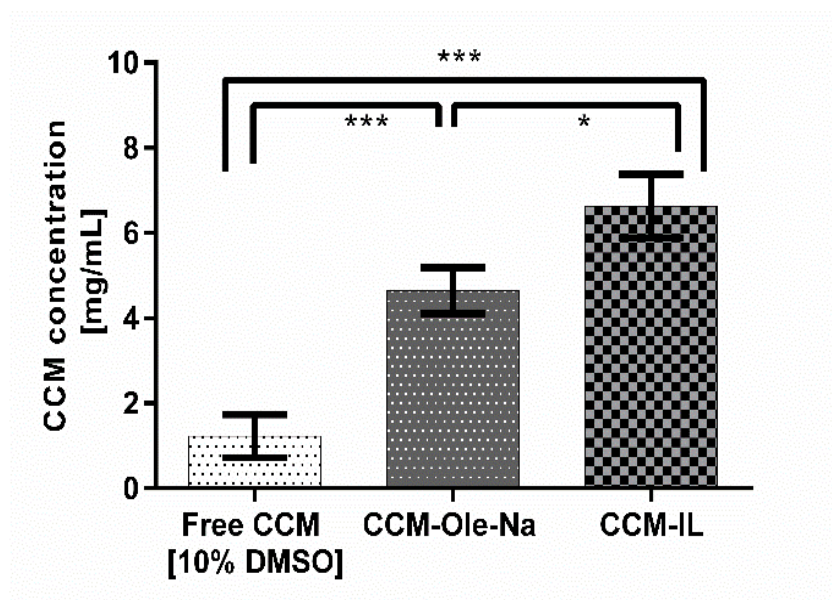


Figure 4-4. Maximum solubility of CCM in water determined by HPLC analysis, when CCM was dispersed in 10% DMSO/water or CCM-IL and CCM-Ole-Na complexes.

Data are shown as mean $\pm$ standard deviation, $n=3$, $*p < 0.05$ and $***p < 0.001$.

The *in vitro* release profiles of CCM from CCM-Ole-Na and CCM-IL complexes were assessed by quantitative HPLC analysis of CCM in the receiving solutions of dialysis systems. Three different media including simulated intestinal fluid (SIF, pH 1.2), simulated gastric fluid (SGF, pH 7.0), and phosphate buffered saline (PBS, pH 7.4) were used as receiving media, representing the different pH and buffering conditions of physiological body fluids. The release of CCM from both complexes showed a biphasic profile, where a burst release was observed within 2–3 h, followed by a sustained release for up to 24 h (see Fig.4-S2 and 4-S3). Almost 80%–90% of the total CCM was released within the first 4 h. After 4 h, the CCM concentration in the simulated medium decreased significantly owing to the autoxidation and degradation of CCM in the presence of water. It was expected that a slower release of CCM would be observed from the CCM-IL complex than from the CCM-Ole-Na complex, owing to the formation of long compact hydrophobic blocks for IL. However, no significant difference between the release profiles was observed. In the acidic medium, the initial burst of CCM was faster than in neutral or basic medium. This may be due to protonation of the complex causing swelling and earlier release of CCM.³³ Encapsulation of CCM by Ole-Na or IL is thought to enhance the aqueous solubility and sustained release of CCM.

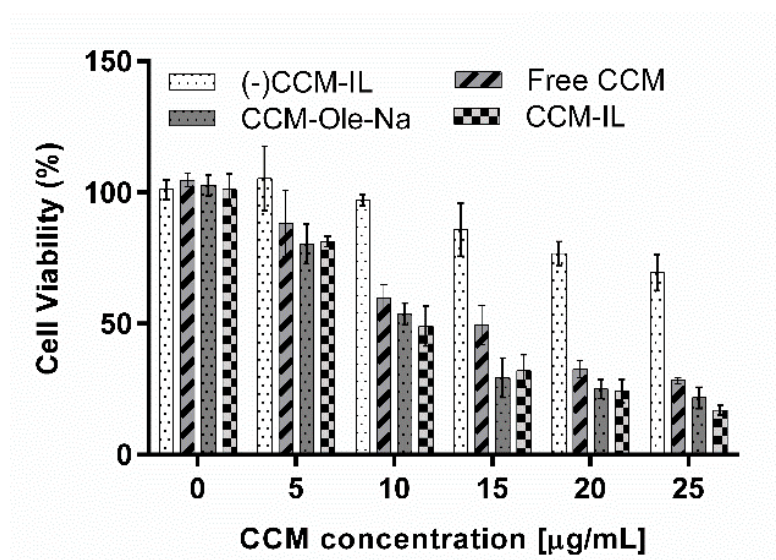


Figure 4-5. In vitro cytotoxicity of CCM-Ole-Na and CCM-IL complexes in HepG2 cells. Data are shown as mean $\pm$ standard deviation, n=3.

The *in vitro* anticancer efficacies of the CCM-Ole-Na and CCM-IL complexes were evaluated by measuring their cytotoxicity, using a human liver carcinoma HepG2 cell line. HepG2 cells were treated with placebo, free CCM, CCM-Ole-Na complex, or CCM-IL complex with various CCM concentrations for 24 h. The cytotoxicity results showed that the cell viability was not significantly affected by treatment with unloaded complexes, indicating the biocompatibility and nontoxicity of the [Ch][Ole] IL and Ole-Na (Fig.4-5). The cation (cholinium group) and anion (oleate group) of the IL are important sources of nutrients and known to be biocompatible and biodegradable.²³ The cell viability of IL was reduced slightly compared with that for Ole-Na, possibly owing to the presence of the cholinium head group in IL, which can cause slightly greater cell membrane penetration than Ole-Na. It has been reported that choline-like structure has high penetration ability through cell membranes.^{23,34} Furthermore, the cell viability significantly decreased in a concentration-dependent manner when cells were exposed to free CCM or the complexes. It has been reported that CCM has the ability to induce *in vitro* cytotoxicity in different cancer cell lines via induction and regulation of important signaling molecules related to apoptosis.^{2,3} Evaluation of cytotoxicity in a normal human cell line (fibroblast cells), showed that both free CCM and the complexes were less toxic to fibroblasts than to HepG2 cells (Fig.4-S4). As CCM has been reported to modulate an important signaling pathway during apoptosis in cancer cells, normal cells remained unaffected. Comparable behavior for free CCM and CCM nanoparticles in normal cells has also been reported previously.^{18,35–38}

4.5 Conclusions

In conclusion, a novel approach for the aqueous formation of CCM encapsulated by a biocompatible [Ch][Ole] IL was demonstrated using a one-step emulsification and rapid freeze-drying method. The resulting CCM-IL complexes could be readily dispersed in water, and showed enhanced solubility, stability, and *in vitro* anti-tumor activity. It is hoped that preclinical and clinical investigations, now ongoing in our laboratory, will reveal the extent of the potential of the complexes.

Supporting Data for Chapter-4

Tables

Table 4-S1. Encapsulation efficiency, yield, half-life ($t_{1/2}$) and conductivity of CCM encapsulated by Ole-Na or [Ch][Ole] IL in water.

Surfactant	Encapsulation Efficiency (%)	Yield (%)	Half-life [$t_{1/2}$, hour]	Conductivity [mS/cm]
Ole-Na	96.4 ± 1.5	86.5 ± 3.9	1.3 ± 1.1	0.011 ± 0.010
[Ch][Ole] IL	97.9 ± 1.2	92.8 ± 2.7	4.3 ± 0.2	0.098 ± 0.010

Data are shown as mean $\pm$ standard deviation, n=3.

Figures

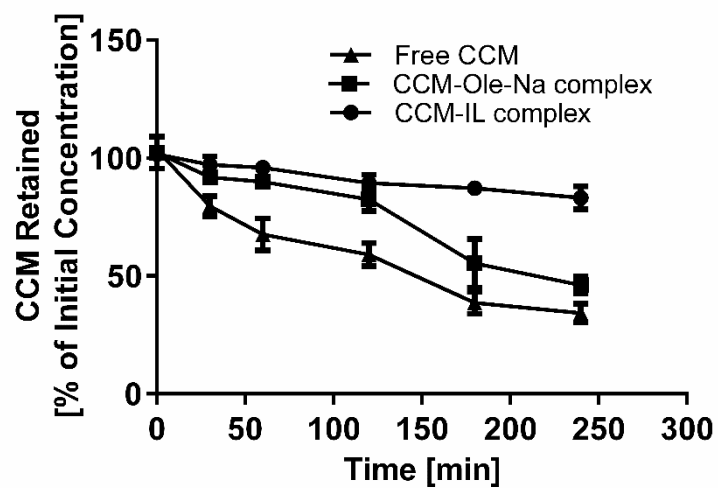


Figure 4-S1. Stability of curcumin in CCM-Ole-Na and CCM-IL complex in water

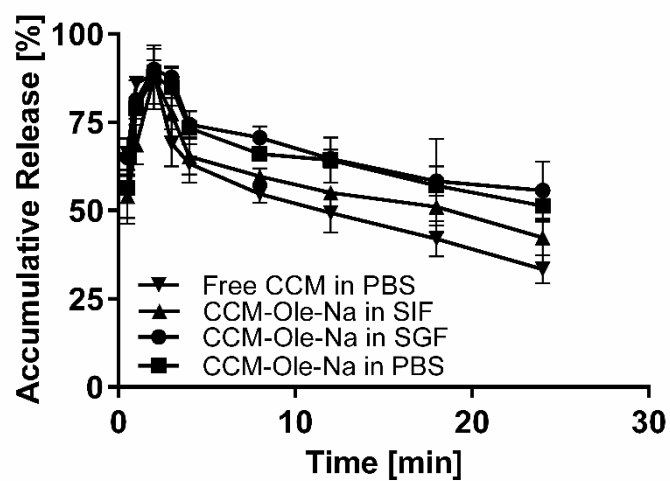


Figure 4-S2. Release profile of CCM-IL complex in different pH of buffered solutions. Data are shown as mean $\pm$ standard deviation, $n=3$.

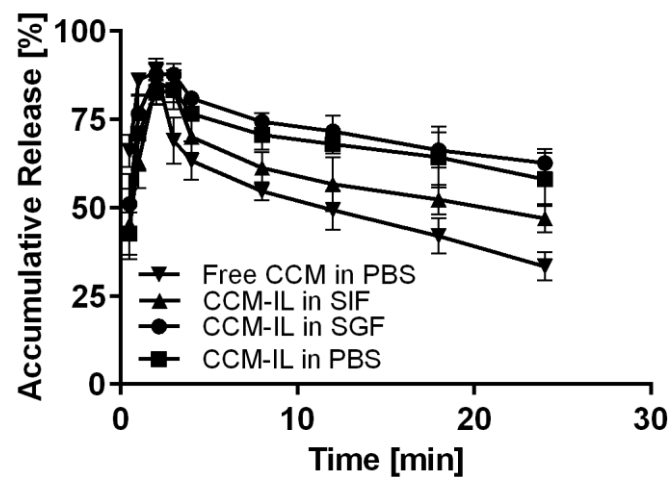


Figure 4-S3. Release profile of CCM-Ole-Na complex in different pH of buffered solutions. Data are shown as mean $\pm$ standard deviation, n=3.

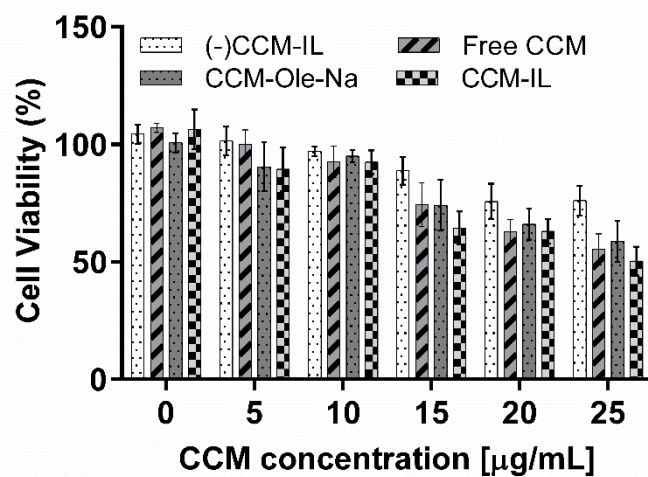


Figure 4-S4. In-vitro cytotoxicity of CCM-Ole-Na or CCM-IL complexes in fibroblast cells. Data are shown as mean $\pm$ standard deviation, n=3.

References

- (1) Sanphui, P.; Goud, N. R.; Khandavilli, U. B. R.; Bhanoth, S.; Nangia, A. New Polymorphs of Curcumin. *Chem. Commun.* **2011**, 47 (17), 5013–5015.
- (2) Yallapu, M. M.; Khan, S.; Maher, D. M.; Ebeling, M. C.; Sundram, V.; Chauhan, N.; Ganju, A.; Balakrishna, S.; Gupta, B. K.; Zafar, N.; et al. Anti-Cancer Activity of Curcumin Loaded Nanoparticles in Prostate Cancer. *Biomaterials* **2014**, 35 (30), 8635–8648.
- (3) Zaman, M. S.; Chauhan, N.; Yallapu, M. M.; Gara, R. K.; Maher, D. M.; Kumari, S.; Sikander, M.; Khan, S.; Zafar, N.; Jaggi, M.; et al. Curcumin Nanoformulation for Cervical Cancer Treatment. *Sci. Rep.* **2016**, 6 (1), 20051.
- (4) Adiwidjaja, J.; McLachlan, A. J.; Boddy, A. V. Curcumin as a Clinically-Promising Anti-Cancer Agent: Pharmacokinetics and Drug Interactions. *Expert Opin. Drug Metab. Toxicol.* **2017**, 13 (9), 953–972.
- (5) Nagahama, K.; Utsumi, T.; Kumano, T.; Maekawa, S.; Oyama, N.; Kawakami, J. Discovery of a New Function of Curcumin Which Enhances Its Anticancer Therapeutic Potency. *Sci. Rep.* **2016**, 6 (30962), 1–14.
- (6) Singh, D.; Chaturvedi, R. K.; Patel, D. K.; Gupta, S. K.; Seth, B.; Bhatnagar, P.; Karmakar, M.; Yadav, A.; Agarwal, S.; Gupta, K. C.; et al. Curcumin-Loaded Nanoparticles Potently Induce Adult Neurogenesis and Reverse Cognitive Deficits in Alzheimer's Disease Model via Canonical Wnt/ β -Catenin Pathway. *ACS Nano* **2013**, 8 (1), 76–103.
- (7) Ho, Y.-C.; Shim, M. S.; Chuang, E.-Y.; Pu, H.-L.; Xia, Y.; Maiti, B.; Liao, Z.-X.; Chiang, W.-L.; Sung, H.-W. Nanoparticles with Dual Responses to Oxidative Stress and Reduced pH for Drug Release and Anti-Inflammatory Applications. *ACS Nano* **2014**, 8 (2), 1213–1221.
- (8) Zhang, D.-W.; Fu, M.; Gao, S.-H.; Liu, J.-L. Curcumin and Diabetes: A Systematic Review. *Evidence-Based Complement. Altern. Med.* **2013**, 2013, 1–16.
- (9) Tayyem, R. F.; Heath, D. D.; Al-delaimy, W. K.; Rock, C. L.; Tayyem, R. F.; Heath, D. D.; Al-delaimy, W. K.; Rock, C. L. Curcumin Content of Turmeric and Curry Powders. *Nutr. Cancer* **2006**, 55 (2), 126–131.
- (10) Pandit, R. S.; Gaikwad, S. C.; Agarkar, G. A.; Gade, A. K.; Rai, M. Curcumin Nanoparticles: Physico-Chemical Fabrication and Its *in Vitro* Efficacy against Human Pathogens. *3 Biotech* **2015**, 5 (6), 991–997.
- (11) Gordon, O. N.; Luis, P. B.; Sintim, H. O.; Schneider, C. Unraveling Curcumin Degradation. *J. Biol. Chem.* **2015**, 290 (8), 4817–4828.
- (12) Moniruzzaman, M.; Kamiya, N.; Goto, M. Ionic Liquid Based Microemulsion with Pharmaceutically Accepted Components: Formulation and Potential Applications. *J. Colloid Interface Sci.* **2010**, 352 (1), 136–142.
- (13) Bhawana; Basniwal, R. K.; Buttar, H. S.; Jain, V. K.; Jain, N. Curcumin Nanoparticles: Preparation, Characterization, and Antimicrobial Study. *J. Agric. Food Chem.* **2011**, 59 (5), 2056–2061.

- (14) Takahashi, M.; Uechi, S.; Takara, K.; Asikin, Y.; Wada, K. Evaluation of an Oral Carrier System in Rats: Bioavailability and Antioxidant Properties of Liposome-Encapsulated Curcumin. *J. Agric. Food Chem.* **2009**, *57* (19), 9141–9146.
- (15) Bisht, S.; Feldmann, G.; Soni, S.; Ravi, R.; Karikar, C.; Maitra, A.; Maitra, A. Polymeric Nanoparticle-Encapsulated Curcumin (“nanocurcumin”): A Novel Strategy for Human Cancer Therapy. *J. Nanobiotechnol* **2007**, *5*, 1–18.
- (16) Yan, H.; Teh, C.; Sreejith, S.; Zhu, L.; Kwok, A.; Fang, W.; Ma, X.; Nguyen, K. T.; Korzh, V.; Zhao, Y. Functional Mesoporous Silica Nanoparticles for Photothermal-Controlled Drug Delivery in Vivo. *Angew. Chemi - Int. Ed.* **2012**, *51* (33), 8373–8377.
- (17) Augello, G.; Emma, M. R.; Craparo, E. F.; Azzolina, A.; Botto, C.; Cavallaro, G.; Di Gaudio, F.; Cervello, M.; Bondi, M. L.; Bachvarov, D. Biocompatible Lipid Nanoparticles as Carriers To Improve Curcumin Efficacy in Ovarian Cancer Treatment. *J. Agric. Food Chem.* **2017**, *65* (7), 1342–1352.
- (18) Razi, M. A.; Wakabayashi, R.; Tahara, Y.; Goto, M.; Kamiya, N. Genipin-Stabilized Caseinatehitosan Nanoparticles for Enhanced Stability and Anti-Cancer Activity of Curcumin. *Colloids Surfaces B Biointerfaces* **2018**, *164*, 308–315.
- (19) Roger, R. D.; Seddon, K. R. Ionic Liquids-Solvents of the Future? *Science (80-.)*. **2003**, *302*, 792–793.
- (20) Hough, W. L.; Rogers, R. D. Ionic Liquids Then and Now: From Solvents to Materials to Active Pharmaceutical Ingredients. *Bull. Chem. Soc. Jpn.* **2007**, *80* (12), 2262–2269.
- (21) Moniruzzaman, M.; Tamura, M.; Tahara, Y.; Kamiya, N.; Goto, M. Ionic Liquid-in-Oil Microemulsion as a Potential Carrier of Sparingly Soluble Drug: Characterization and Cytotoxicity Evaluation. *Int. J. Pharm.* **2010**, *400* (1–2), 243–250.
- (22) Adawiyah, N.; Moniruzzaman, M.; Hawatulaila, S.; Goto, M. Ionic Liquids as a Potential Tool for Drug Delivery Systems. *MedChemComm.* The Royal Society of Chemistry October 12, 2016, pp 1881–1897.
- (23) Kulkarni, A. R.; Mehrara, B. J.; Pusic, A. L.; Matros, E.; McCarthy, C. M.; Disa, J. J. Effect of Choline Carboxylate Ionic Liquids on Biological Membranes. *Colloids Surf B Biointerfaces* **2016**, *32* (3), 178–182.
- (24) Liu, X.; Ji, C.; Yang, Q.; Bao, Z.; Fan, X.; Yang, Y.; Xing, H. Carboxylate Ionic Liquids Combining Low Cytotoxicity toward HepG2 Cell and High Separation Efficiency for Bioactive Molecules. *ACS Sustain. Chem. Eng.* **2017**, *5* (2), 1974–1981.
- (25) Morandeira, L.; Álvarez, M. S.; Markiewicz, M.; Stolte, S.; Rodríguez, A.; Sanromán, M. Á.; Deive, F. J. Testing True Choline Ionic Liquid Biocompatibility from a Biotechnological Standpoint. *ACS Sustain. Chem. Eng.* **2017**, *5* (9), 8302–8309.
- (26) Vassiliou, E. K.; Gonzalez, A.; Garcia, C.; Tadros, J. H.; Chakraborty, G.; Toney, J. H. Oleic Acid and Peanut Oil High in Oleic Acid Reverse the Inhibitory Effect of Insulin Production of the Inflammatory Cytokine TNF- Both *in Vitro* and *in Vivo* Systems. *Lipids Health Dis.* **2009**, *8* (25), 1–10.
- (27) He, Q.; Liu, Z.; Wu, Y.; Shao, Y.; Sun, J.; Jiang, L.; Wang, A.; Lu, Z.; Wang, W. Oleic Acid Induces Apoptosis and Autophagy in the Treatment of Tongue Squamous Cell Carcinomas. *Sci. Rep.* **2017**, *7* (1), 11842.

- (28) Eunok, C.; David, B. M. Mechanisms of Antioxidants in the Oxidation of Foods. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8* (4), 345–358.
- (29) Carrillo, C.; Cavia, M. D. M. D. M.; Alonso-Torre, S. Role of Oleic Acid in Immune System; Mechanism of Action; a Review. *Nutr. Hosp.* **2012**, *27* (4), 978–990.
- (30) Jin, W.; Yang, Q.; Zhang, Z.; Bao, Z.; Ren, Q.; Yang, Y.; Xing, H. Self-Assembly Induced Solubilization of Drug-like Molecules in Nanostructured Ionic Liquids. *Chem. Commun.* **2015**, *51* (67), 13170–13173.
- (31) Mu, L.; Shi, Y.; Ji, T.; Chen, L.; Yuan, R.; Wang, H.; Zhu, J. Ionic Grease Lubricants: Protic [Triethanolamine][Oleic Acid] and Aprotic [Choline][Oleic Acid]. *ACS Appl. Mater. Interfaces* **2016**, *8* (7), 4977–4984.
- (32) Kharat, M.; Du, Z.; Zhang, G.; McClements, D. J. Physical and Chemical Stability of Curcumin in Aqueous Solutions and Emulsions: Impact of PH, Temperature, and Molecular Environment. *J. Agric. Food Chem.* **2017**, *65* (8), 1525–1532.
- (33) Menon, D.; Anitha, A.; Nair, S. V.; Deepagan, V. G.; Jayakumar, R.; Divya Rani, V. V. Preparation, Characterization, *in Vitro* Drug Release and Biological Studies of Curcumin Loaded Dextran Sulphate–Chitosan Nanoparticles. *Carbohydr. Polym.* **2011**, *84* (3), 1158–1164.
- (34) Kawai, K.; Kaneko, K.; Kawakami, H.; Yonezawa, T. Bioinspired Choline-like Ionic Liquids: Their Penetration Ability through Cell Membranes and Application to Sem Visualization of Hydrous Samples. *Langmuir* **2011**, *27* (16), 9671–9675.
- (35) Kunwar, A.; Barik, A.; Mishra, B.; Rathinasamy, K.; Pandey, R.; Priyadarsini, K. I. Quantitative Cellular Uptake, Localization and Cytotoxicity of Curcumin in Normal and Tumor Cells. *Biochim. Biophys. Acta - Gen. Subj.* **2008**, *1780* (4), 673–679.
- (36) Chang, R.; Sun, L.; Webster, T. J. Short Communication: Selective Cytotoxicity of Curcumin on Osteosarcoma Cells Compared to Healthy Osteoblasts. *Int. J. Nanomedicine* **2014**, *9* (1), 461–465.
- (37) Syng-Ai, C.; Kumari, A. L.; Khar, A. Effect of Curcumin on Normal and Tumor Cells: Role of Glutathione and Bcl-2. *Mol. Cancer Ther.* **2004**, *3* (9), 1101–1108.
- (38) Kong, Y.; Ma, W.; Liu, X.; Zu, Y.; Fu, Y.; Wu, N.; Liang, L.; Yao, L.; Efferth, T. Cytotoxic Activity of Curcumin towards CCRF-CEM Leukemia Cells and Its Effect on DNA Damage. *Molecules* **2009**, *14* (12), 5328–5338.

Nomenclature and Abbreviations for Chapter-4

Abbreviation	Name/description
CCM	Curcumin
IL	Ionic Liquid
CCM-IL complex	Curcumin-Ionic Liquid complex
[Ch][Ole] IL	Choline Oleic Acid Ionic Liquid
Ole-Na	Oleic Acid Sodium Salt
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide

CHAPTER-5

SUMMARY AND FUTURE WORK

5.1 Summary

In the past several years, ILs have been at the cutting edge of the most promising research and technology in the scientific field. The applications of ILs not only found to be covered the classical areas of knowledge but also expended as an important candidates to solve critical problems such as clean and efficient energy. In particular, ILs were found to play a special role in the pharmaceutical industry as clean and multifunctional solvents for a variety of applications. This neoteric class of solvents, ILs, generally presents interesting properties, namely, negligible vapor pressure at relatively ambient conditions, high thermal, chemical, and electrochemical stability, and broadly tunable properties with regard to polarity, hydrophobicity, and solvent miscibility. These properties result from a matchless combination of molecular characteristics of their constitutive ions. Moreover, many types of ILs can be regarded as nanosegregated fluids with polar networks permeated by apolar domains, which enables the understanding of their peculiar solvent behavior at a molecular level and the numerous applications to solve classical problems addressing today's societal challenges. One of the major challenge of the pharmaceutical research is the increasing number of active pharmaceutical ingredients (APIs) possessing limited aqueous solubility in biopharmaceuticals classification system class II drugs. The challenging aspects of new formulations of such drug molecules are associated with their slow dissolution in biological fluids, and thus insufficient and inconsistent systemic exposure and consequent suboptimal clinical efficacy. ILs have been considered as solvents and/or materials in the field of pharmaceuticals with the possibility of improved performance such as improved solubility, stability and drug delivery. In fact, due to their tailor-made properties, ILs can dissolve complex molecules including biologically active compounds and it is highly desirable for ILs to be potential solvents for overcoming the problems concomitant with pharmaceutical industries.

In **chapter-2**, we preliminary reported an IL-based paclitaxel (PTX) formulation that composed PTX/cholinium amino acid ILs/ethanol/tween-80/water. A significant enhancement in the solubility of PTX was observed with considerable correlation with the density and viscosity of the ILs, and with the side chain of the amino acids used as anions in the ILs. Moreover, the formulations were stable for up to 3 months. The driving force for the stability of the formulation was hypothesized to be the involvement of different types of interactions between the IL and PTX. *In vitro* cytotoxicity and anti-tumor activity for the IL-based

formulations were evaluated using HeLa cells. The IL vehicles without PTX were found to be less cytotoxic than cremophor EL-based formulation (Taxol), while both the IL-based PTX formulation and Taxol exhibited a similar antitumor activity. Finally, *in vitro* hypersensitivity reactions were evaluated using THP-1 cells and found to be significantly lower with the IL-based formulation than Taxol. This study demonstrated that specially designed ILs could provide a potentially safer alternative to Cremophor EL as an effective PTX formulation for cancer treatment giving fewer hypersensitivity reactions.

In **chapter-3**, as a follow-up to previous study, further investigation of the IL-based PTX formulation was reported where *in vivo* biocompatibility, pharmacokinetics, antitumor activity, and hypersensitivity were evaluated using mouse model with Taxol as a reference. In this study, the stability of the IL-PTX formulation was monitored by quantitative HPLC analysis, which showed that IL-PTX was more stable at 4 °C than at room temperature. The *in vivo* study showed that the IL-PTX formulation could be used in a therapeutic application as a biocompatible component of a drug delivery system. To assess the *in-vivo* biocompatibility, IL or IL-mediated formulations were administered intravenously by maintaining physiological buffered conditions (neutral pH and isotonic salt concentration). From *in vivo* pharmacokinetics data, the IL-PTX formulation was found to have a similar systemic circulation time and slower elimination rate compared to Taxol. Furthermore, *in vivo* antitumor and hypersensitivity experiments in C57BL/6 mice revealed that IL-PTX had similar antitumor activity to Taxol, but a significantly smaller hypersensitivity effect compared with Taxol. Therefore, the IL-mediated formulation has potential to be an effective and safe drug delivery system for PTX.

In **chapter-4**, we reported a one-step emulsification and rapid freeze-drying process to develop a curcumin–ionic liquid (CCM–IL) complex that could be readily dispersed in water with a significantly enhanced solubility and stability (half-life) compared to that of free CCM. For this study, the IL consisted of choline as the cation and oleic acid as the anion used as a surfactant. Both the cation (choline) and anion (oleic acid) in the IL are widely accepted as biocompatible one. Furthermore, oleic acid has been reported to have many interesting biological functions including anti-inflammatory, anti-carcinogenic, anti-oxidative, and immune system activation properties. The developed CCM-IL complex could be readily dispersed in water with a significantly enhanced solubility of ~8 mg/mL and half-life ($t_{1/2}$) of ~260 min compared with free CCM (solubility ~30 nM and $t_{1/2}$ ~20 min). Comparison with a similar complex encapsulated using oleic acid sodium salt (Ole-Na), led to the conclusion that

both the cation and anion in the IL have significant contributory roles in the solubilization and stabilization of CCM in the complex. Furthermore, release studies in three different physiological fluids showed bi-phasic profiles with an initial burst release of CCM over a few hours, followed by slow sustained release up to 24 h. Finally, the anticancer activity of the CCM-IL complex was found to be significantly higher when used to treat cancer cells (HepG2) compared with normal cells (fibroblast). The emulsification and freeze-drying process using an IL consisting of a long chain carbon backbone, as a surfactant, may provide an alternative way of enhancing the solubility of poorly water-soluble compounds. Therefore, combining the therapeutic effects of the surfactant and drug considered as a novel approach for the preparation of surfactant–drug complexes.

5.2 Future work

The development of pharmaceutical applications using IL-based methodologies requires a deep understanding of ILs both in terms of their macroscopic properties and also at the molecular level, because structural aspects have been shown to play a crucial and unexpected role in a large number of situations. Despite the fact that ILs can no longer be considered a new field, the large diversity of combinations of cations and anions producing novel ILs with new specific properties is astonishing. Despite the fact that the conventional pharmaceutical industry is based on solids and solids processing, the use of ILs should be pushed forward because it might be beneficial to many of the drugs that have been put on hold owing to their limited aqueous solubility or polymorphic conversion, thus bringing a new market value for these products. Further attention should be focused on the large-scale production of biocompatible and biodegradable ILs. Research into these issues is underway, and we confidently believe that new types of biocompatible and biodegradable ILs will be available to promote the IL- based drug delivery technology.

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