**Design and Synthesis of Ibuprofen and Ketoprofen Conjugates with Gemcitabine as a Possible Mutual Prodrugs**

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**Abstract**

**Introduction**

Cyclooxygenase type-2 (COX-2) overexpressed in some cancers and plays a pivotal role throughout oncogenesis and here we exploit the rationale to explore the use of NSAID (ibuprofen and ketoprofen) in combination with current anticancer drug gemcitabine for the prevention and/or treatment of cancer together with targeting of gemcitabine to these cancer tissues.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed classes of drugs and their long-term regimens have been greatly shortened due to their gastrointestinal side effects. Masking of carboxyl group of these drugs, by conjugating them with gemcitabine through their carboxyl, could reduce these side effects.

**Aim of study**

The study describes design and synthesis of mutual ester pro-drug of NSAID and gemcitabine, which is designated to generate the complementary pharmacological action as a single chemical entity with improved drug targeting and reducing side effects.

**Method**

Esterification of the two entities of the resultant compound was done by using dicyclohexylcarbodiimide (DCC) as catalyst. The carboxyl group of the NSAIDs was activated by DCC in dimethylformamide (DMF) as solvent, followed by reaction of NSAID, through its activated carboxyl, with gemcitabine, through its hydroxyl, by stirring the MDF solution of the first with the chloroform solution of the latter. Purification was done by recrystallization (by cold water and then by ethanol) after removal of the main side product by filtration. The reactions and synthesis were conferred by FT-IR, CHNS, physiochemical properties, melting point and thin layer chromatography (TLC).

**Results and conclusion**

In the present study, the combined derivatives of NSAIDs (ibuprofen and ketoprofen) with anticancer anti metabolite drug (gemcitabine) was successfully designed and synthesized as possible new mutual pro-drugs and with acceptable purity. It is expected to enhance oral bioavailability and targeting of gemcitabine and improve therapeutic action on specific targets in addition to gastrointestinal side effects of NSAIDs.

**Key Words:**

Gemcitabine, ibuprofen, ketoprofen, mutual prodrugs, cancer, and NSAIDs.

**Introduction**

Gemcitabine (2′,2′-difluorodeoxycytidine) a nucleoside analogue, is approved as a first-line combination chemotherapeutic agent for treatment of pancreatic, non-small cell lung and breast cancer, and second-line therapy for treatment of ovarian cancer. Gemcitabine undergoes intracellular metabolism by nucleoside kinases to form the active diphosphate and triphosphate nucleosides leading to inhibition of DNA synthesis [1]. Gemcitabine is rapidly metabolized by the so-called cytidine-deaminase which limits its efficacy. Because of extensive deamination by intestinal cells, its oral administration results in very low bioavailability [2]. In order to achieve the required concentration over sufficient periods of time, repeated application of relatively high doses is required [3]. This, in turn, leads to dose-limiting systemic toxicity. The plasma half-life after intravenous infusion is 8- 17 min in human plasma. Therefore, it is required in high doses. Furthermore, Gemcitabine is a highly hydrophilic molecule with log P value 1.4. Till now, there is no oral formulation of Gemcitabine HCl in the market. It is available in the market in the freeze-dried form of an aqueous solution of the HCl salt known as Gemzar. After reconstitution Gemzar is used for intravenous administration as an infusion only [4].

NSAIDs are used extensively to alleviate inflammation, pain, rheumatoid arthritis, and osteoarthritis. Long-term regimens of NSAIDs have been greatly shortened due to their gastrointestinal side effects [5, 6]. They are prone to produce certain prevalent side effects such as gastrointestinal irritation though these are more likely with high doses and prolonged use [7]. Owing to their wide spread consumption, a large population taking NSAIDs is reported to eventually develop gastric ulcers and related complications, leading to a condition popularly known as NSAID gastropathy, which is characterized by sub-epithelial hemorrhages, erosions and ulcers. Around 50% of patients are reported to have gastric erosions and 10-30% suffer from gastric ulcer [8]. Introduction of more potent agents with an even greater propensity for toxic side effects increased the awareness about NSAID-induced gastro-duodenal ulceration and provided impetus for development of effective NSAIDs with more favorable GI safety profile [9].

NSAIDs produce stomach irritation, which in patients with preexisting conditions or patients taking large amounts of NSAIDs for extended periods may be severe. This irritation is associated in part with the presence of an acidic functionality in these agents. The carboxylic acid functionality commonly found in these agents is un-ionized in the highly acidic environment of the stomach. As a result, these agents are more lipophilic in nature and may pass into the cells of the gastric mucosa. The intracellular pH of these cells is more basic than that of the stomach lumen, and the NSAID becomes ionized. This results in backflow of from the lumen into these cells, with concomitant cellular damage. This type of damage could be reduced if the carboxylic acid function could be reversibly masked in these agents [10].

Recent human epidemiological studies suggest an inverse relationship between intake of NSAIDs and the risk of colorectal cancer [11], and the severity or incidence of Alzheimer’s disease [12]. (NSAIDs) induce apoptosis in a variety of cancer cells, including those of colon, prostate, breast and leukemia. In addition, the classical NSAIDs sulindac and aspirin are promising chemopreventive agents against colon cancer. NSAIDs inhibit cyclooxygenases (COX) preventing the formation of prostaglandins, prostacyclin and thromboxane. NSAIDs also exert other biological effects (table-1), including generation of reactive oxygen species (ROS) and inhibition of NF-κB-mediated signals [13, 14]. Several recent observations cast doubt on the idea that COX is the sole target of NSAID action. The finding that some NSAIDs can inhibit proliferation and induce cell death in cells that do not express COX, suggests that other targets may play a part in NSAIDs-mediated apoptosis [14].

The neutralization of the carboxylate of NSAIDs can generate COX-2- selective inhibitors. Esterification or amidation of NSAIDs abolishes COX-1 inhibitory activity while maintaining COX-2 inhibitory activity. Because many NSAIDs contain a carboxylic acid group, this would represent a general strategy for the conversion of nonselective NSAIDs into selective COX-2 inhibitors. [16, 17] SAR analysis reveals that structurally diverse functionalities can serve as part of the ester or amide linkage in indomethacin, resulting in highly selective COX-2 inhibitors. [18]

Numerous experimental, epidemiologic, and clinical studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs), particularly the highly selective cyclooxygenase (COX)-2 inhibitors, have promise as anticancer agents [19]. Several epidemiological studies have reported that individuals who regularly use aspirin and other NSAIDs have a lower incidence of adenomatous polyps and lower incidences of or deaths from colorectal cancer compared with nonusers [20]. NSAIDs may also be associated with reduced risk of cancers of bladder, breast, oesophagus, lung, ovary, prostate, stomach, liver, pancreas, tongue and glioblastoma multiforme [21].

NSAIDs may have attractive effects other than analgesic and anti-inflammatory effect, these include: The novel NSAID derivatives as potential prodrugs for anticancer therapy or chemopreventive applications with less toxic side effects have been synthesized. It has been proved that phosphoramidate derivatives of fenoprofen (fig 1.6), ketoprofen, ibuprofen, indomethacine and diclofenac possess significantly higher antiproliferative activities [22].

In the early 1990s, it was discovered that COX exists in two forms, COX-1 and COX-2. Within the cell, both isoforms are located on the endoplasmic reticulum and the nuclear envelope. While COX-1 is constitutively expressed in most tissues, COX-2 is inducible by trauma, tumor promoters, growth factors and inflammatory cytokines [23]. COX-1 is the only COX isoform expressed in platelets and gastric mucosa of normal humans. COX-1 produces PGs that regulate essential physiologic functions such as gastric mucosal protection, maintenance of normal kidney function, and platelet aggregation. COX-1 expression can be increased only two- to four- fold under most circumstances. In contrast, COX-2 is usually barely detectable during normal physiologic conditions. It is an immediate-early gene induced upon cell activation and stimulation by pathophysiological stimuli, and it can be rapidly induced to increase PG production ten- to eighty-fold [24]. Various laboratory studies suggested that NSAIDs reduce the risk of colon cancer and that inhibition of colon carcinogenesis is mediated through modulation of prostaglandin by COX isoenzyme. Over expression of COX-2 has been observed in colon tumors therefore specific inhibitors of COX-2 could potentially serve as chemopreventive agents. A recent study with celecoxib and nimesulide in intestinal polyps in mice and colonic aberrant crypt foci (ACF) formation in rats induced by azoxymethane indicated that both agents possess strong chemopreventive activity against colon carcinogenesis [25].

Several lines of evidence have demonstrated that COX-2 plays an important role in tumor development and progression. First, COX-2 has been reported to be upregulated in a variety of malignant tumors including colon, gastric, pancreatic and breast cancers [26-29], and high-level expression of COX-2 in tumor tissue is related to poor prognosis in several tumor types including lung cancer, breast cancer and gliomas [30-32]. Further, studies in carcinogen-induced tumors and genetically modified animals have shown that non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors have profound suppressive effects on tumor development [33, 34]. Finally, the overexpression of COX-2 is sufficient to cause tumorigenesis in animal models, and the deletion of the COX-2 gene suppresses tumor progression in mice predisposed to intestinal neoplasia [35]. These findings thus provide compelling evidence that COX-2 is an obligatory player in human cancers. Epidemiological studies indicated that the regular use of aspirin can reduce the risk of esophageal cancer up to 90% [36, 37].

COX-2 was consistently and more intensely observed in metastatic lesions compared with the corresponding primary tumor. In general, COX-2 is expressed in 40% to 80% of neoplastic cells in human cancers and the extent and intensity of expression is greater in cancerous than in noncancer cells. Moreover, well- and moderately-differentiated cancers have significantly higher COX-2 expression than poorly differentiated cancers. COX-2 is also detected in noncancerous cells immediately adjacent to tumor cells and in the angiogenic vasculature within tumors and in pre-existing blood vessels adjacent to tumors [38]. In contrast, COX-2 is not detected in the vasculature of normal tissues [39]. The COX-2 activity may be responsible for increased prostaglandin levels in cancer tissues [40, 41]. Importantly, recent work demonstrates a relationship between overexpression of COX-2 and the invasiveness and survival of patients with breast [42], colon, [43-45] gastric, [46, 47] and lung [48] cancers may benefit from treatment with celecoxib. Additionally, combination therapy with celecoxib and other molecular targeted agents such as aromatase inhibitors (ie, exemestane) or agents that block HER-2/neu activation may also prove beneficial in clinical trials of breast cancer.

**Aim of the work**

The aim of this work is to design and synthesis of ibuprofen-gemcitabine ester conjugate (figure 1) and ketoprofen-gemcitabine ester conjugate (figure 2) as possible mutual prodrugs. The proposed compounds are outlined below:



**Figure 1: Ibuprofen-gemcitabine ester-conjugate**



**Figure 2: Ketoprofen-gemcitabine ester conjugate**

Prodrug design is a choice of approach in solving many of the stability, solubility, permeability, adverse effects and targeting problems that plague drug discovery and development. The prodrug approach has the ability to keep promising new drug candidates alive through development and improving the safety and efficacy of existing drug products. It is effective for drugs suffering from undesirable side effects. A mutual prodrug normally comprises of two biologically active agents coupled together so that each acts as a pro-moiety for the other agent [26, 49].

In this study we designed mutual prodrugs ddepending on the following principles (which are mentioned above in details):

1. Derivatization of the carboxylate moiety in NSAIDs would eliminate their ability to inhibit COX-1 without significantly affecting their COX-2 inhibitory properties.
2. Gastrointestinal side effects constitute the most frequent of all the adverse reactions of NSAIDs and often these reactions lead to GIT ulceration and hemorrhage. Therefore, considerable attention has been focused in the development of bio-reversible derivatives, by temporarily masking the acidic group of NSAIDs, as a promising mean of reducing or abolishing the GI toxicity.
3. Interestingly, substantial experimental and clinical evidence indicates a role for NSAIDs in the prevention of various types of cancer, especially when combined with chemotherapy.
4. Based on this observation, the designated mutual prodrug expected to improve the complementary pharmacological and enhanced physiochemical properties (oral delivery system of gemcitabine comparing to the low bioavailability of the parent gemcitabine).
5. Epidemiological studies provided the first evidence that COX may be involved in the pathogenesis of cancer. Several reports indicate NSAIDs can prevent the development of various human tumors including colon, breast, lung, gastric, and esophageal neoplasias.
6. In general, COX-2 is expressed in 40% to 80% of neoplastic cells in human cancers and the extent and intensity of expression is greater in cancerous than in noncancerous cells. Moreover, well and moderately-differentiated cancers have significantly higher COX-2 expression than poorly differentiated cancers. In contrast, COX-2 is not detected in the vasculature of normal tissues, so, the mutual prodrugs of NSAIDs-gemcitabine are oriented to targeting of gemcitabine selectively to carcinogenic cells.

**Materials and Methods**

**Materials**

All reagents and anhydrous solvents were of analytical grade type and used as received from the commercial suppliers (Merck, Germany; Reidel De-Haen, Germany; Sigma-Aldrich, Germany and BDH, England). Ibuprofen, ketoprofen, and gemcitabine were supplied by the Wauxi Hexia chemicals Company, china.

**Method of Synthesis of Ibuprofen-Gemcitabine Ester Conjugate**

412.6 mg of ibuprofen (2 mmol) was dissolved in 20 ml of chloroform followed by addition of 412 mg of N,N'-Dicyclohexylcarbodiimide DCC (2 mmol). The reaction mixture was stirred at room temperature for 1 hour (solution A). 600 mg of Gemcitabine HCl (2mmol) and 20 mg of dimethylaminopyridine (DMAP) were dissolved in 30 ml DMF (solution B). Then solution A and B were mixed and stirred for 48 hour at room temperature (scheme 1). The precipitate of N,N′-Dicyclohexylurea DCU was filtered off and solvent of filtrate was removed under reduced pressure. Cold water (90 ml) was added to the obtained residue and the precipitate was collected and recrystallized from absolute ethanol. Yield is 58%. Physicochemical properties, FTIR spectra, and CHN elemental microanalysis are shown in tables 1, 2, and 3 respectively.

**Method of Synthesis of Ketoprofen-Gemcitabine Ester Conjugate**

508.6 mg of ketoprofen (2 mmol) was dissolved in 20 ml of chloroform followed by addition of 412 mg of N,N'-Dicyclohexylcarbodiimide DCC (2 mmol). The reaction mixture was stirred at room temperature for 1 hour (solution A). 600 mg of Gemcitabine HCl (2mmol) and 20 mg of dimethylaminopyridine (DMAP) were dissolved in 30 ml DMF (solution B). Then solution A and B were mixed and stirred for 48 hour at room temperature (scheme 2). The precipitate of N,N′-Dicyclohexylurea DCU was filtered off and solvent of filtrate was removed under reduced pressure. Cold water (90 ml) was added to the obtained residue and the precipitate was collected and recrystallized from absolute ethanol.Yield is 61%. Physicochemical properties, FTIR spectra, and CHN elemental microanalysis are shown in tables 1, 2, and 3 respectively.

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**Scheme 1: Synthesis of ibuprofen-gemcitabine ester conjugate**

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**Scheme 2: Synthesis of ketoprofen-gemcitabine ester conjugate**

**Methods of Analysis of Compounds**

Melting points (table 1) were determined by capillary method on electrical melting point apparatus SMP30 Stuart, England. To check the purity and progress of reactions, ascending thin layer chromatography (TLC) was run on Merk aluminum silica plates (0.2 mm). The identification of compounds was done using a U.V. detector and the chromatograms were eluted with chloroform: methanol (8.5:1.5) (table 1). IR spectra were recorded on a FTIR-spectrophotometer Shimadzu as KBr disks (table 2). CHNS microanalysis (table 3) was done using a Euro EA 3000 elemental analyzer (Italy).

**Results and Discussion**

Physicochemical properties (Chemical formula, Molecular weight, % yield, Melting point Cᵒ, and Rf values) of the resulted compounds are listed in (table 1) below, while FTIR spectra and elemental microanalysis results of the same compounds are listed in (table 2) and (table 3) respectively.

**Table 1: Physicochemical properties of the resulted compounds.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Rf values***  | ***Melting point Cᵒ***  | ***Description***  | ***% yield***  | ***Molecular weight***  | ***Chemical formula***  | ***Compounds***  |
| 0.64  | 135-138  | White crystals  | 58  | 486.5 | C22H27ClF2N3O5  | **Ibuprofen-gemcitabine ester conjugate** |
| 0.77  | 161-165 | White crystals  | 61  | 534.5 | C25H23Cl1F2N3O6  | **ketoprofen-gemcitabine ester conjugate** |

**Table 2: FTIR spectra of the resulted compounds.**

|  |  |
| --- | --- |
| ***Main changes in FTIR spectra*** | ***Compounds***  |
| 1. Absence of broad band of O-H stretching vibration of carboxyl of ketoprofen (center at 3000 cm-1) due to esterification.
2. Appearance of strong band of C=O stretching vibration of synthesized ester at 1738 cm-1
3. Appearance of two bands of C-O stretching vibrations of synthesized ester at 1065 cm-1 and 1220 cm-1
4. Appearance of strong and somewhat sharp band of O-H stretching vibrations of secondary hydroxyl of gemcitabine at 3535 cm-1.
5. Appearance of strong band of C=O stretching vibration of amide of gemcitabine at 1655 cm-1
6. Appearance of strong bands of C-F stretching vibrations of synthesized ester at 1115 cm-1.
 | **Ibuprofen-gemcitabine ester conjugate** |
| 1. Absence of broad band of O-H stretching vibration of carboxyl of ketoprofen (center at 3000 cm-1) due to esterification.
2. Appearance of strong band of C=O stretching vibration of synthesized ester at 1746 cm-1.
 | **ketoprofen-gemcitabine ester conjugate** |

**Continued Table 2: FTIR spectra of the resulted compounds.**

|  |  |
| --- | --- |
| ***Main changes in FTIR spectra*** | ***Compounds***  |
| 1. Appearance of two bands of C-O stretching vibrations of synthesized ester at 1085 cm-1 and 1230 cm-1.
2. Appearance of strong and somewhat sharp band of O-H stretching vibrations of secondary hydroxyl of gemcitabine at 3590 cm-1.
3. Appearance of strong band of C=O stretching vibration of amide of gemcitabine at 1650 cm-1

Appearance of strong bands of C-F stretching vibrations of synthesized ester at 1100 cm-1. | **Ketoprofen-gemcitabine ester conjugate** |

**Table 3: CHN elemental microanalysis of the resulted compounds.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **%N** | **%H** | **%C** | **Chemical formula** | **Compounds**  |
| **observed** | **calculated** | **observed** | **calculated** | **observed** | **calculated** |
| 8.14 | 8.63 | 5.17 | 5.55 | 51.63 | 54.27 | C22H27ClF2N3O5  | **Ibuprofen-gemcitabine ester conjugate** |
| 6.74 | 7.86 | 3.69 | 4.3 | 54.01 | 56.13 | C25H23Cl1F2N3O6  | **ketoprofen-gemcitabine ester conjugate** |

Pharmacological studies consistently demonstrate that COX-2 inhibitors hinder tumor growth and metastasis in dose-dependent manner in various animal models. Importantly, several investigators have shown that COX-2 inhibitors may act additively or synergistically with currently used cytotoxic and molecularly targeted agents. There is a growing evidence that COX-2 plays a pivotal role throughout oncogenesis which explore the rational use of COX-2 inhibitors for the prevention and/or treatment of cancer as a single agent or in combination with current anticancer modalities.

Compound synthesized in this study were designed to be mutual prodrugs in which two therapeutic compounds bonded via a cleavable covalent chemical linkage. Derivatization of the carboxylate moiety in NSAIDs would eliminate their ability to inhibit COX-1 without significantly affecting their COX-2 inhibitory properties and the development of bio-reversible ester derivatives, by temporarily masking the acidic group of NSAIDs, as a promising mean of reducing or abolishing the GI toxicity.

Gemcitabine is administered by the intravenous route, since it is extensively metabolized by the gastrointestinal tract, so when coupled with NSAID as single chemical entity in an ester mutual pro-drug it is expected to enhance oral bioavailability of the parent gemcitabine since it will be unavailable for the destructive means in the GIT. It is expected that uptake of gemcitabine is increased by the effected tumor that overexpress COX-2 if treated by these synthesized mutual prodrugs.

Dicyclohexylcarbodiimide (DCC) is the most commonly used coupling reagents (figure 3). It is a dehydrating agents, which abstract a molecule of water from the carboxyl and hydroxyl groups of two reactants, which gives rise to the very insoluble N,N′-dicyclohexylurea, the N-acyl-N,N′-dicyclohexylurea, and the O-acyl-N,N′-dicyclohexylisourea. The first step in carbodiimide-mediated reactions is the addition of the reagent (DCC) to the carboxyl group of ibuprofen and ketoprofen to give the O-acylisourea derivatives which are a transient intermediate as shown in figure 3.



**Figure 3: The mechanism of amide and ester bond formation by N,Nʹ-dicyclohexylcarbodiimide-promoted condensation of a carboxylic acid and an amine or alcohol**

The O-acylisourea is highly activated, reacting with a hydroxyl groupe in the presence of DMAP to produce an ester (figure 4). The carboxyl group is activated by treating with N,N′-dicyclo hexylcarbodiimide (DCC), where the addition of alcohol is followed to form an ester. The acid is first added to the C=N bond of DCC, and nucleophilic acyl substitution by amine or alcohol is done .The method that used 5-10% of 4-dimethylaminopyridine (DMAP) as a catalyst with DCC called the Steglich esterification which was first described by Wolfgang Steglich. DMAP role in Steglich esterification, acting as an acyl transfer-reagent in a manner shown in figure 4.

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**Figure 4: DMAP role in Steglich esterification**

The reaction conditions could be deviated toward esterification rather than amide bond formation, when both amine and hydroxyl groups are present in the same molecule, by protecting amine group. Using of gemcitabine as hydrochloride salt will reduce the reactivity of secondary amine of gemcitabine toward such reaction, so that only hydroxyl group will be available as a good nucleophile which will attach the activated carboxyl of NSAIDs to form ester bond.

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