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Synthesis and Biological Evaluation of Paclitaxel and Camptothecin Prodrugs on the Basis of 2-Nitroimidazole

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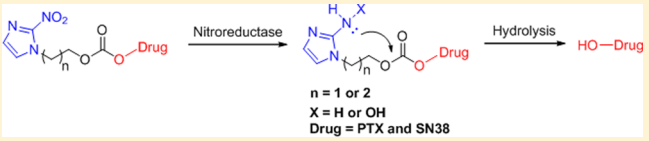
Supporting Information

ABSTRACT: Due to the low esterase activity in human plasma, many ester and carbonate prodrugs tested in humans may be less effective than that in preclinical animals. In this letter, PTX and SN-38 were attached to the *N*-1 position of 2-nitroimidazole via a carbonate linker. Presumably, 2-aminoimidazole may help promote the intramolecular hydrolysis of the carbonate bond. The prodrugs exhibited a considerable stability in buffers at different pH values as well as in human plasma. Furthermore, a rapid reduction was exhibited in the presence of nitroreductase. An *in vitro* cytotoxicity assay demonstrated that hypoxic conditions could increase the toxicity of prodrugs. Potentially, the compound species may form a new class of promising antitumor agents.



KEYWORDS: *2-Nitroimidazole, paclitaxel, SN-38, prodrug*

Paclitaxel (PTX) and camptothecin (CPT) represent the most important anticancer drugs in clinical use to date and exhibit a high antitumor efficacy against a wide range of tumor species.[1,2] Paclitaxel is a microtubule stabilizing agent isolated from *Taxus brevifolia*. Among others, paclitaxel and its analogue docetaxel are used for the treatment of solid tumors, including ovarian, breast, and lung cancesr.[3−6] Camptothecin is a potent antitumor alkaloid isolated from *Camptotheca acuminate* and was found to target DNA topoisomerase I. Camptothecin derivatives such as 10-hydroxycamptothecin, topotecan, and irinotecan (active metabolite: SN-38) are used for the treatment of ovarian and colorectal cancers.[7−10] Unfortunately, these compounds exhibit significant side effects on healthy tissues, poor solubility characteristics in aqueous media, and multidrug resistance. Ultimately, these drawbacks limit the applicability of the drugs in clinical treatments. In an effort to overcome these drawbacks, the development of prodrugs represents a common approach offering a site-specific release.[11−14]

A wide field of research studies has demonstrated that the lactone ring in camptothecin represents a critical feature for antitumor activity, corresponding to topoisomerase I inhibition. However, the instability of the lactone ring in the plasma decreases antitumor efficiency.[15] Masking 20-OH by transforming it to the corresponding water-insoluble alkyl ester or carbonate has been shown to increase lactone stability in the plasma compared with their parent compounds.[16] Some prodrugs conjugated with PEG in 20-OH position have been demonstrated to increase the solubility in aqueous media.[24] However, one of the most common problems of such prodrugs is that they have been shown to be too stable in preclinical studies, ultimately not releasing the active compound at sufficiently high concentrations. Furthermore, insufficient prodrug conversion may reduce therapeutic efficacy due to the notion that the active compound may never reach a therapeutically relevant concentration. Overall, this issue may also occur in the case of ester paclitaxel prodrugs in 2′-OH position, representing a common strategy for paclitaxel prodrug design.[17] A large body of research studies has shown that human plasma esterase activity is markedly different from the esterase activity in other animal species resulting in pharmacokinetic differences of these prodrugs. The efficiency of prodrugs tested in humans may be lower than that in preclinical animals, including mouse and rat, due to the lower esterase activity in human plasma.[18,19] Therefore, we believe that it is necessary to introduce a trigger for bond cleavage in 20-OH position of camptothecin and in 2′-OH of paclitaxel in order to ensure active drug release at the target site.

Hypoxia, a deficiency in the amount of oxygen reaching the tissues, occurs in a variety of human disease states including solid tumors.[20,21] Among others, the presence of hypoxia in solid tumors results in chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, and resistance to cell death.[22,23] Considerable effort has been put forward to develop bioreductive prodrugs, which could be selectively activated under hypoxic microenvironments.[24−27] Denny et al. have reported the use of prodrugs of cyclization-activated aromatic mustard species involving an *ortho*-nitrophenyl group.[28] Ono et al. have reported a hypoxia-activated pro-oligo compounds based on 3-(2-nitrophenyl)propan-1-ol with a phosphoester bond activated via intramolecular cyclization of 2-aminophenol.[29] For the latter study, 2-

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nitroimidazole was demonstrated to represent an improved bioreductive group compared to nitrobenzene.[30] Therefore, we designed a new class of prodrugs in *N*-1 position of 2-nitroimidazole instead of the commonly used C-5 position. Here, PTX and SN-38 were conjugated to 2-(2-nitro-1*H*-imidazol-1-yl)ethanol and 3-(2-nitro-1*H*-imidazol-1-yl)propan-1-ol via a carbonate bond. We further found that 2-aminoimidazole derivatives have the ability to promote the hydrolysis of the carbonate bond and induce the release of the prodrugs.[31] Moreover, we investigated two 2-nitroimidazole derivatives containing ethanol and propanol in *N*-1 position of 2-nitroimidazole. These prodrugs were designed with significantly increased activity at hypoxic tumor sites. One of the most important findings of this study was that the drug release mechanism via 2-aminoimidazole increased the drug release efficiency (Figure 1).

The prodrugs were synthesized according to the process outlined in Scheme 1. 2-(2-Nitro-1*H*-imidazol-1-yl)ethanol and 3-(2-nitro-1*H*-imidazol-1-yl)propan-1-ol were activated by addition of 4-nitrophenyl carbonochloridate and were then reacted with PTX to afford **2C-PTX** and **3C-PTX**. Conversely, 10-*O*-*tert*-butyldimethylsilyl-SN-38 was treated with triphosgene and then reacted with 2-(2-nitro-1*H*-imidazol-1-yl)ethanol and 3-(2-nitro-1*H*-imidazol-1-yl)propan-1-ol. The TBS group was deprotected by addition of TBAF to afford **2C-SN38** and **3C-SN38** (Scheme 1).

The stability of the compounds was tested first as this factor seems to be critical for biological evaluation. The compounds were evaluated in buffer at different pH conditions (pH 5, pH 6.5, and pH 7.4) for a period of 48 h using HPLC (Figure 2). The results indicated that the half-life ($t_{1/2}$) value of **3C-SN38** and **2C-PTX** in buffer at pH 5 was 40 and 48 h, respectively. The $t_{1/2}$ values of **2C-SN38** and **3C-PTX** were found to be beyond 48 h. In buffer at pH 6.5, the half-life value of **2C-SN38**, **2C-PTX**, and **3C-PTX** was 41, 24, and 24 h, respectively, and the $t_{1/2}$ of **3C-SN38** under these conditions was found to be beyond 48 h. In buffer at pH 7.4, the $t_{1/2}$ value of **2C-PTX** and **3C-PTX** was 28 and 36 h, respectively, and the $t_{1/2}$ values of **2C-SN38** and **3C-SN38** were beyond 48 h. We concluded that these compounds were stable in different buffers at different pH values. We then tested the compounds *in vitro* in mouse and human plasma. It was known that the much higher level of esterase in mouse plasma compared to human plasma may accelerate the hydrolysis of the carbonate bond. The results obtained indicate that the test compounds degrade rapidly in mouse plasma. The half-life of **2C-SN38**, **3C-SN38**, **2C-PTX**,

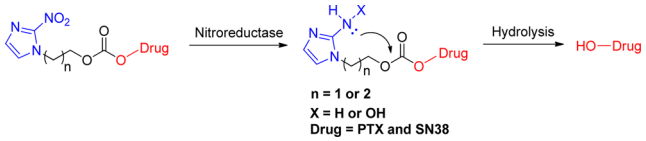
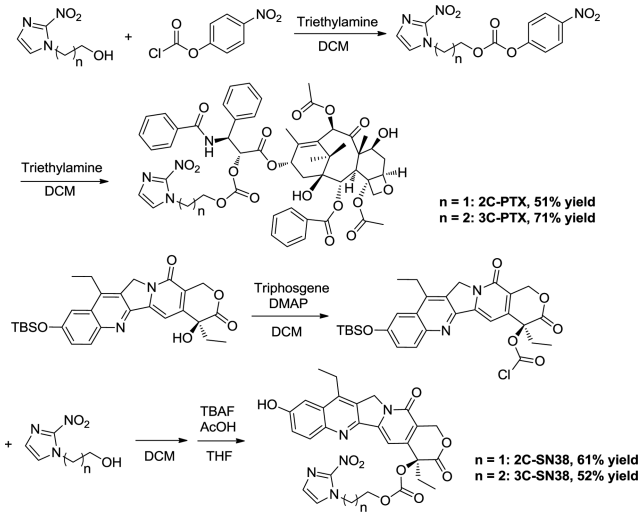


Figure 1. Proposed mechanism of prodrug activated via bioreduction.

and **3C-PTX** in mouse plasma was determined to be 47, 37, 20, and 55 min, respectively. However, the metabolic degradation rates of the compounds in human plasma were found to be much slower. The $t_{1/2}$ of **2C-SN38**, **3C-SN38**, **2C-PTX**, and **3C-PTX** was 6, 2, 2, and 6 h. The compounds exhibited a considerable stability in buffers at different pH values and human plasma, a finding that is consistent with the other reports found in the literature.[12,32] Our results indicate that the

Scheme 1. Synthetic Route of Prodrugs



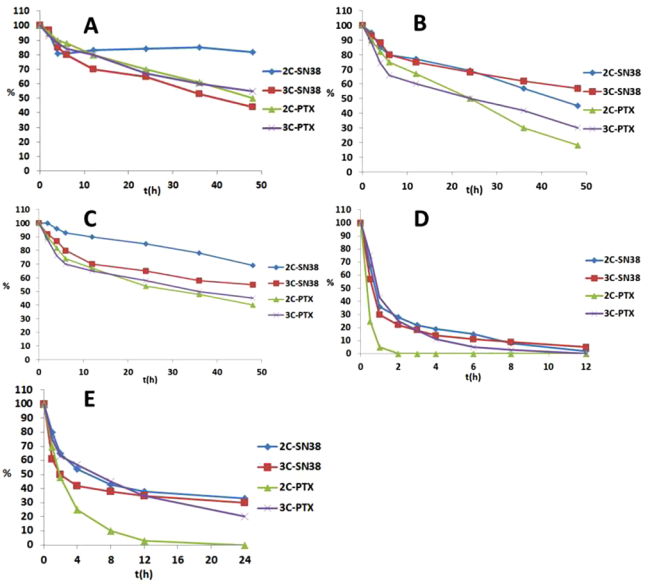


Figure 2. Stability of prodrugs in different buffer: (A) pH = 5.0 ABS; (B) pH = 6.5 PBS; (C) pH = 7.4 PBS; (D) mouse plasma; (E) human plasma.

carbonate bond seems to be sufficiently stable in human plasma, a critical feature for the future development of bioreductive prodrug species.

To determine whether the prodrug fragments provide the desired active agent as intended, we carried out a chemical reduction over Pd/C in the presence of H_2 in THF at 37 °C. The individual fragments were characterized by LC/MS. Compound **3C-SN38** afforded the best results and was found to be entirely reduced, resulting in the generation of two products, namely, SN-38 and the 2-aminoimidazole intermediate of **3C-SN38**. The mass peak corresponding to the 2-aminoimidazole intermediate could be found in the MS-ESI spectrum (m/z = 561.02 $[M + H]^+$) (Figure 3). Compound **2C-SN38** was determined to be mainly reduced to a byproduct, resulting in almost negligible release of SN-38. Presumably, the amino group is too close to the lactone ring ultimately causing a side reaction. However, the characterization results using the PTX derivatives were complex. Both the 2-aminoimidazole

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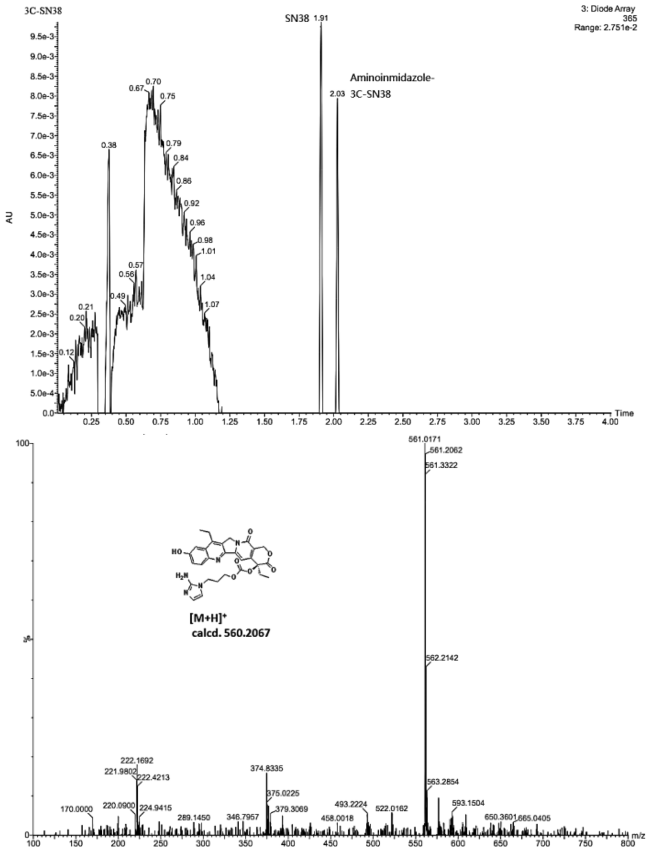

Figure 3. LC/MS charts of **3C-SN38** after chemical reduction over Pd/C and H_2 in THF.

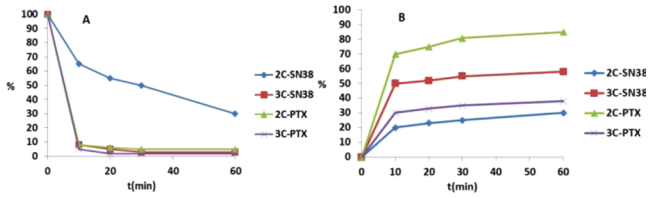

Figure 4. Percent of prodrug remaining (A) and active drug releasing (B) in 1 h of incubation with nitroreductase in the presence of NADPH.

intermediate and PTX could be detected, while some unknown derivatives were found to be generated simultaneously. We believe that the prodrugs of PTX were not stable under the reductive conditions used here (Figure S1).

In hypoxic tumors, the reductase level has been reported to be much higher than in normal tissues. Presumably, this feature provides an opportunity to specifically target hypoxia. For the further investigation of the activate mechanism of the prodrugs, nitroreductase (NTR) extracted from *Escherichia coli* was used. The prodrugs (10 μM, 1% DMSO) dissolved in PBS (pH 7.4) were incubated with nitroreductase (50 μg/mL) and NADPH (1 μmol/mL) at 37 °C and the biological reduction process was monitored by HPLC (Figure 4). The prodrugs were found to be rapidly reduced by nitroreductase, except for **2C-SN38**, which reacted slower. The $t_{1/2}$ of **2C-SN38** was determined to be 30 min, and the $t_{1/2}$ values of all other compound species were within 10 min. Approximately 30% of **2C-SN38** still remained after 1 h and only 30% of the active drug was found to be released. Presumably, **2C-SN38** was too stable under these bioreductive conditions described. Considering the results for **3C-SN38** and **3C-PTX**, even though the prodrug disappeared rapidly, the generation rates of the active drug were not as fast as the compounds reduced. Therefore, we believe that the 2-aminointermediates, as well as some byproducts, may have been generated. However, **2C-PTX** was determined to be the most suitable compound, with an overall PTX conversion rate of 85% within 1 h. Likely, the bioreductive process via nitroreductase was somewhat milder, resulting in a reduced formation of **2C-PTX** byproducts compared to chemical reduction. The 2-aminoimidazole intermediate of **2C-PTX** was not detected, which may indicate that the drug release process of the 2-aminoimidazole intermediate under physiological conditions was very fast.

The compounds were evaluated for their cytotoxicity characteristics under normoxic and hypoxic (94% N_2, 5% CO_2, 1% O_2) conditions *in vitro* using H460 human lung cancer cells and HT29 human colon cancer cells. These cell lines are known to express high levels of oxygen-insensitive reductase, potentially activating these prodrugs via enzymatic reduction and increasing the toxicity of the prodrugs under hypoxic conditions, and were therefore used for cytotoxicity tests. The cells were incubated in the presence of the test compounds at various concentrations for 72 h under normoxic or hypoxic conditions. The cell viability and proliferation behavior were assessed by MTT. The IC_{50} values for the proliferation inhibition of the tested compounds are shown listed in Table 1. The results obtained indicate that **3C-SN38** exhibited the highest selectivity ratio of 2.03 in the H460 cell line and **2C-PTX** exhibited the highest selectivity ratio of 3.11 in the HT29 cell line. All tested compounds featured moderate selectivity toward hypoxic tumor cells. The hypoxic conditions could increase the toxicity of prodrugs.

In summary, a new class of carbonate prodrugs on the basis of 2-nitroimidazole was studied in order to release the active drug in an efficient manner. The prodrugs exhibited a considerable stability in different buffer and human plasma.

Table 1. *In Vitro* Cytotoxicity Assay Data Summary

	H460			HT29		
	IC_{50} (μM)			IC_{50} (μM)		
	AIR	N_2	$IC_{50}(AIR)/IC_{50}(N_2)$	AIR	N_2	$IC_{50}(AIR)/IC_{50}(N_2)$
SN-38	0.050 ± 0.001	0.047 ± 0.007	1.06	0.096 ± 0.048	0.119 ± 0.018	0.82
PTX	0.006 ± 0.001	0.007 ± 0.005	0.86	0.014 ± 0.002	0.015 ± 0.001	0.93
2C-SN38	0.091 ± 0.022	0.063 ± 0.008	1.44	>10	9.56 ± 0.66	>1.05
3C-SN38	0.201 ± 0.022	0.099 ± 0.012	2.03	>10	6.02 ± 0.52	>1.67
2C-PTX	0.009 ± 0.001	0.006 ± 0.002	1.5	0.140 ± 0.041	0.045 ± 0.035	3.11
3C-PTX	0.021 ± 0.003	0.018 ± 0.003	1.17	0.290 ± 0.112	0.159 ± 0.038	1.82

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The compounds were found to be reduced rapidly in the presence of nitroreductase. Among the studied compounds, compound **2C-PTX** was determined to release 85% of the active drug, the highest conversion rate under the studied bioreduction conditions. Four prodrugs exhibited moderate hypoxia selectivity in H460 and HT29 cell lines. On the basis of the results obtained, we hypothesize that these prodrugs may find potential future applications as antitumor agents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00189.

Experimental procedures, characterization data, and NMR spectra (PDF)

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Author Contributions

‡These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

SN-38, 7-ethyl-10-hydroxy-camptothecin; NADPH, nicotinamide adenine dinucleotide phosphate

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