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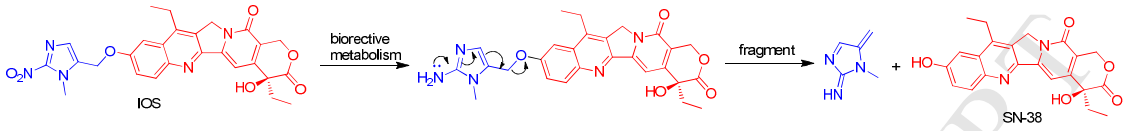
Graphical Abstract

In this study, we proposed a new hypoxia-activated prodrug that conjugated (1-methyl-2-nitro-1H-imidazol-5-yl)methanol with SN-38. Compound IOS containing ether linkage was evaluated as a promising hypoxia-selective antitumor agent.



Synthesis and biological evaluation of hypoxia-activated prodrugs of SN-38

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Abstract:

We designed new hypoxia-activated prodrugs by conjugating (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol with 7-ethyl-10-hydroxy camptothecin (SN-38). Initially, we improved the method of multi-gram scale synthesis of (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol, which increased the yield to 42% compared to 8% by the original synthesis method. The improved method was used to synthesize evofosfamide (TH-302) and hypoxia-activated prodrugs of SN-38. Two different linkages between (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol and SN-38 were evaluated that afforded different hypoxia-selectivity and toxicity. Compound **16** (IOS), containing an ether linkage, was considered to be a promising hypoxia-selective antitumor agent.

Keywords: Hypoxia-activated prodrug, SN-38, 2-Nitroimidazole

1. Introduction

Natural products are an important resource in the search for new anticancer molecules. Many compounds derived from plant metabolites are commonly used in chemotherapy, such as camptothecin, paclitaxel, and teniposide. However, the severe side effects on normal tissues and multidrug resistance limit their applications in clinical treatment.[1,2]

Camptothecin is a potent antitumor alkaloid that is isolated from *Camptotheca acuminate*, and it specifically targets DNA topoisomerase I (Topo I). Topo I is a DNA unwinding protein. Camptothecin inhibits the catalytic activity of Topo I and blocks the rejoining step of the

breakage-reunion reaction of unwound DNA.[3-5] Structure–activity relationship studies of camptothecin derivatives summarized that a hydroxyl substitution at the 10-position, such as topotecan and irinotecan, enhanced antitumor activity.[6] Irinotecan, acting through its active metabolite SN-38, was used in the treatment of metastatic colorectal cancer. However, non-specific toxicity and poor water solubility were the major clinical limitations of camptothecin derivatives. To improve these deficiencies, many efforts have been made to develop new camptothecin analogs, such as peptidic and boronic acid prodrugs, nanodrugs, and antibody conjugates of SN-38, some of which have been recently tested in clinical trials.[7-16] Recently, Bertozzi *et al*. reported that camptothecin derivatives could impair the hypoxia-induced cell transcriptional response and reduce hypoxia-inducible factor-1α (HIF-1α) protein expression and activity,[17] which indicated that camptothecin could be used to treat hypoxic tumors. Thus far, there have been few studies on this application of camptothecin. Of note, Zhang *et al*. reported a novel class of camptothecin derivatives conjugated with 4-nitrobenzyl and 4-nitrofuryl alcohols at the 20-hydroxyl position for use in hypoxia-targeting tumor chemotherapy.[18]

Hypoxia occurs in many human diseases, especially in solid tumors.[19,20] Hypoxia in solid tumors has led to multiple contributions to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, and resistance to cell death.[21-24] Because of its critical role in tumor progression and resistance to radiation and chemotherapy, hypoxia is becoming a compelling therapeutic target.[25] Hypoxia-activated prodrugs (HAPs), which are selectively activated by reductive enzymes with a bioreductive group, have been proposed.[26-31] 2-Nitroimidazole has been widely used in the development of hypoxia-selective agents.[32-35] One of the most clinically advanced hypoxia-activated prodrugs, evofosfamide (TH-302), was synthesized on the basis of the DNA cross-linking toxin bromo-isophosphoramide mustard (Br-IPM) and (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol.[36] However, TH-302 did not show effective results in phase III trials. Patients with locally advanced unresectable or metastatic soft tissue sarcoma treated with TH-302 in combination with doxorubicin did not demonstrate a statistically significant improvement in overall survival compared to those observed with doxorubicin alone (ClinicalTrials.gov Identifiers: NCT01746979; NCT01440088).

In this study, we designed new hypoxia-activated prodrugs of SN-38 for the first time. We initially developed an improved method for multi-gram scale synthesis of

(1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol, which increased the yield to 42% compared with 8% by the original synthetic method. This method was then used for to synthesize TH-302 and hypoxia-activated prodrugs of SN-38. SN-38 has a phenolic hydroxyl group that can be used to attach functional groups. Attaching antitumor drugs to the functional groups with a suitable bond significantly affected the pharmacokinetic properties of the prodrugs. Therefore, we used ether and bis-carbamate linkages to connect (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol and SN-38[37,38]. These prodrugs were designed to be stable in the bloodstream and normal tissues. At the target site, the prodrugs underwent different reductive processes, causing the release of SN-38 (**Figure 1**). We aimed to reduce the toxicity of SN-38 to normal tissues and increase the effectiveness against treatment-resistant hypoxic tumor cells.

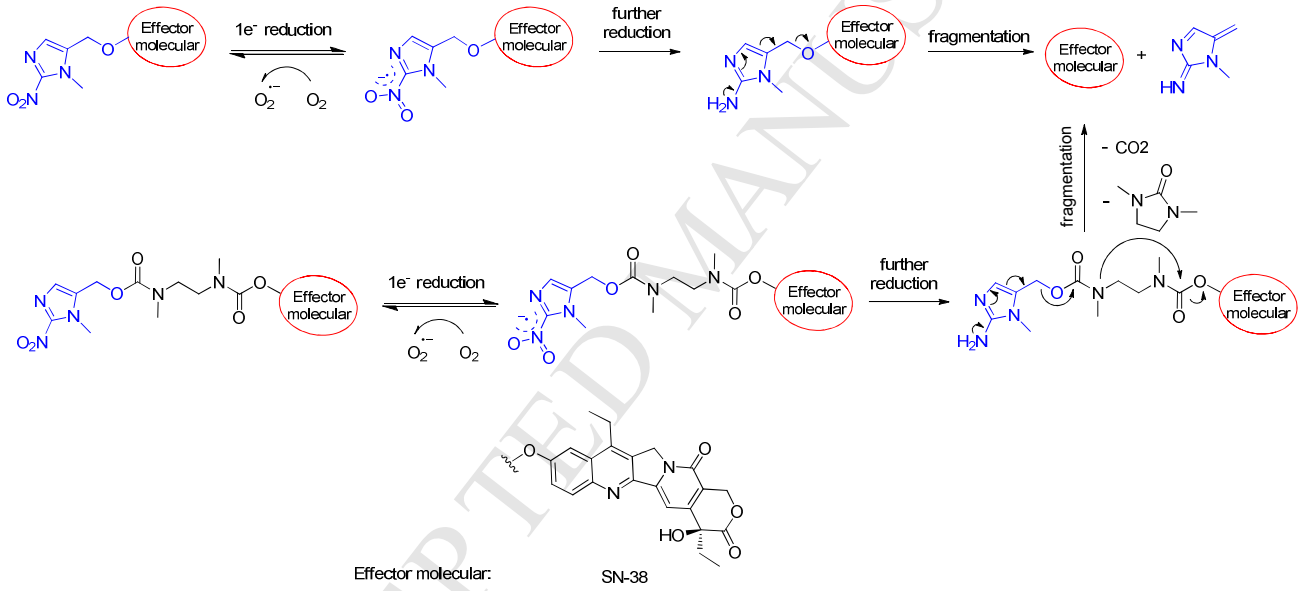


Figure 1. Proposed mechanism of prodrugs activated via enzymatic metabolism under hypoxic conditions.

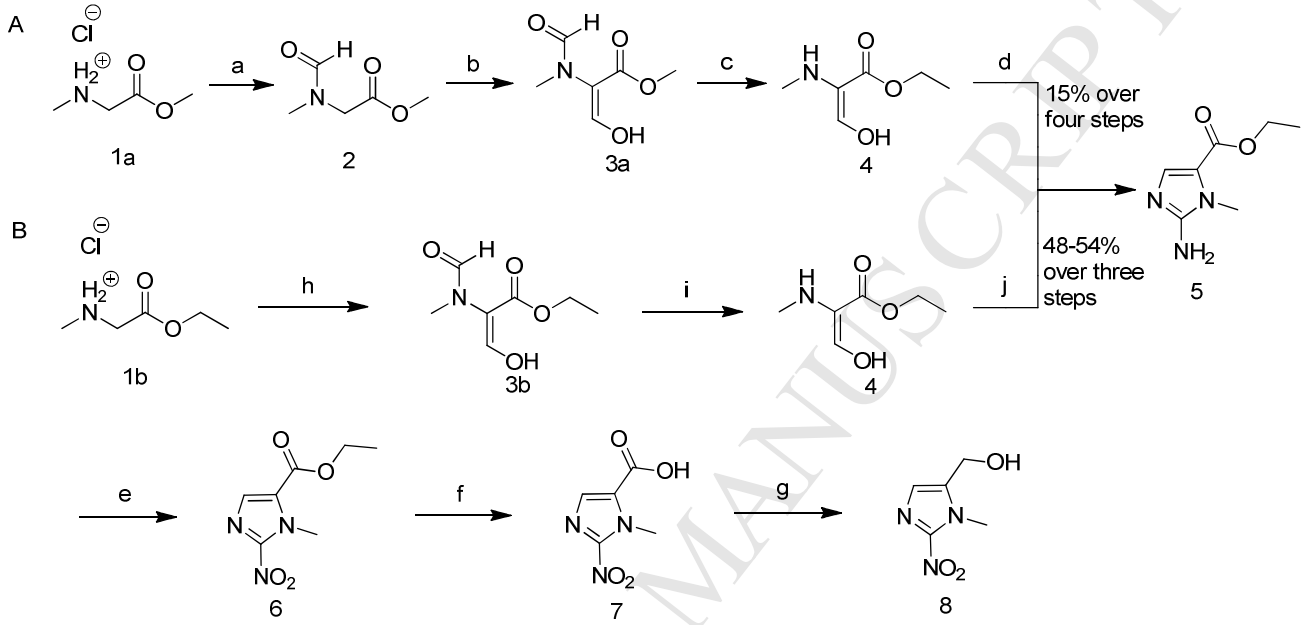
2. Results and discussion

2.1. Chemistry

The original synthesis method of (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol, which was developed by Matteucci *et al.*, started with a sarcosine methyl ester HCl salt and led to a final product over seven steps with an 8% overall yield.[39] (**Scheme 1, A**). It was obvious that the first four steps produced a very low yield. Thus, recently, O'Connor *et al.* reported an improved method for this synthesis (**Scheme 1, B**).[40] They improved the synthesis of compound **5** with a one-pot procedure, with yields of 48–54% over the first three steps. However, there were several problems

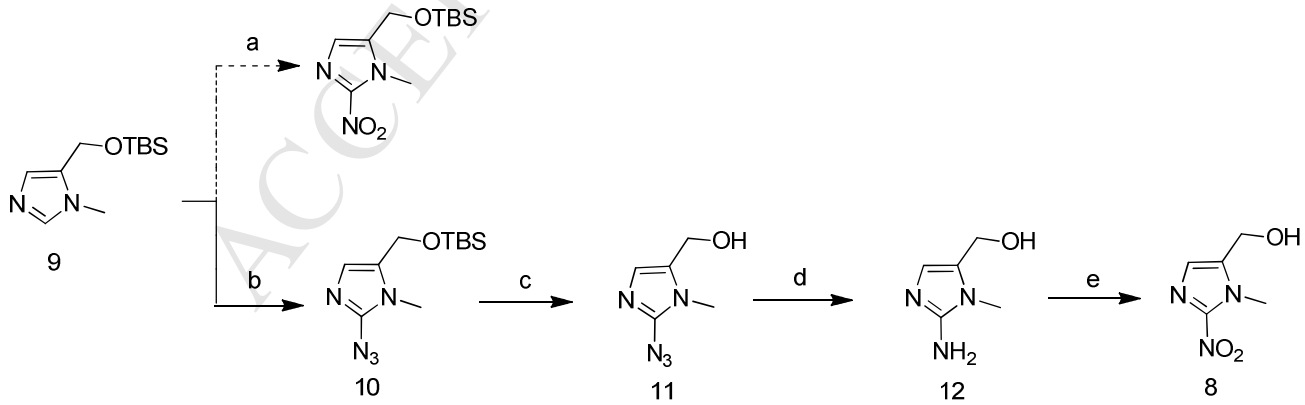
when we investigated this procedure. The insolubility of the starting material and the large amount of NaH consumed (as much as 10 equivalents) made it extremely difficult to scale up. It was initiated with 2 g of starting material and yielded several hundred milligrams of compound **8**. The amount produced was far from sufficient.

Scheme 1 Original synthesis



Reagents and conditions: A. a) K_2CO_3, HCOOEt, EtOH; b) NaH, HCOOEt; c) HCl, EtOH; d) NH_2CN, AcONa, H_2O, 15% over four steps; e) AcOH, $NaNO_2$, 62%;f) NaOH, H_2O, 95%; g) isobutyl chloroformate, $NaBH_4$, THF, 88%. **B.** h) HCOOEt, THF, NaH; i) EtOH, conc. HCl; j) EtOH, H_2O, NH_2CN, 48–54% over three steps.

Scheme 2 New synthesis



Reagents and conditions: a) n-BuLi, i-$PrONO_2$, THF; b) n-BuLi, TsN_3, THF, 82%; c) HCl, MeOH, 4h, 87%; d) Pd/C, H_2, MeOH, 1h, 96%; e) $NaNO_2$, Cu, HBF_4, H_2O, 61%.

For the aforementioned reasons, we considered a new strategy to obtain the basic structure of

imidazole (**Scheme 2)**. It has been reported that 5-(((tert-butyldimethylsilyl)oxy)methyl)-1-methyl-1*H*-imidazole (compound **9)** was readily obtained by using the commercially available 1,3-dihydroxyacetone dimer in three steps upon scale of up to tens of grams.[41-46] Compared with the original synthesis, this synthetic route was more convenient to obtain a 1-methyl-5-hydroxymethylimidazole scaffold at a considerable yield.

Initially, we sought to introduce the nitro functional group into starting material **9** in one step. Davis *et al*. reported a convenient method for the synthesis of 2-substituted imidazole that was based on the reaction of 2-lithioimidazole with electrophiles.[47] Thus, 2-lithioimidazole was formed by the reaction of compound **9** with n-BuLi at -78°C in anhydrous THF and was followed by dropwise addition of i-PrONO_2. However, no reaction was observed at –78°C. Then, the reaction became complicated when the temperature was raised to -40°C. No target product was obtained. As a result, i-PrONO_2 was unsuitable for introducing the nitro group.

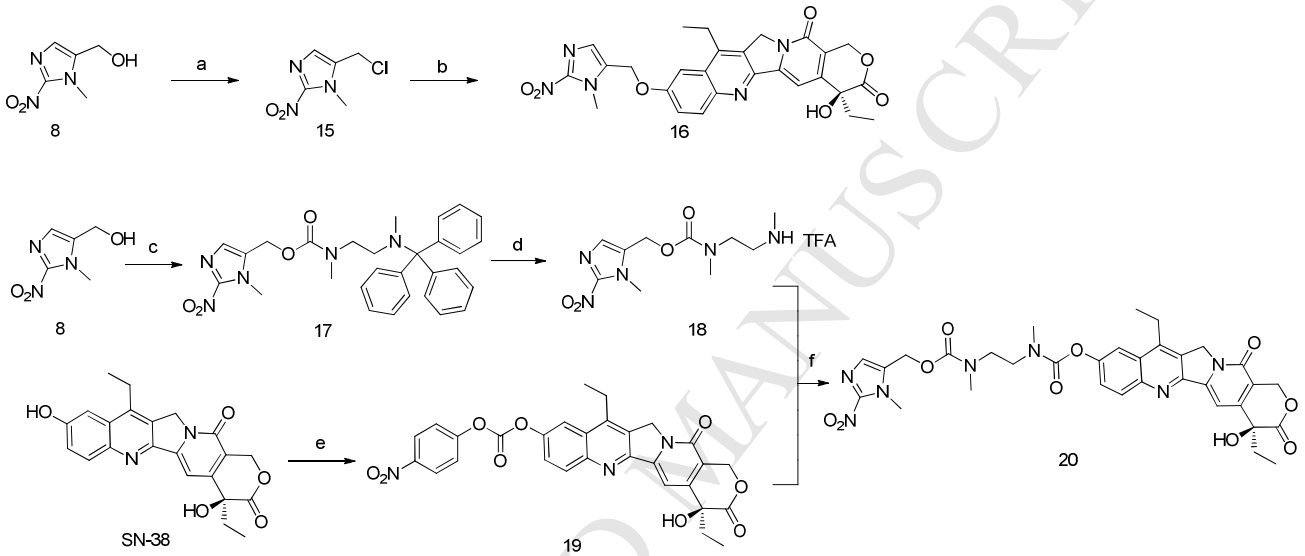
There were few examples of successfully introducing a nitro group directly at the C-2 position of imidazole derivatives. Therefore, we investigated another strategy that used TsN_3 to introduce the azido group instead.[48] 2-Lithioimidazole was obtained as before, and TsN_3 was added to it. The reaction was completed rapidly and purified by silica gel to produce compound **10** with an 82% yield. Then, compound **10** was deprotected with HCl/MeOH and purified with ether to give compound **11**. Compound **11** was reduced by Pd/C and H_2 to produce amino **12**. With multiple grams of amino **12** in hand, we sought to introduce a nitro functional group via diazotization, which was the most critical process in this method. The original synthesis, which used acetic acid, provided a low yield. We considered that diazonium acetate was less stable than diazonium tetrafluoroborate. Therefore, HBF_4 (40%) was used instead of AcOH. At the same time, Cu powder as a catalyst was necessary for the reaction.[49] These modified conditions improved diazotization, with a moderate yield of 61%. Compound **8** was produced with a moderate yield of 3 g.

TH-302 was synthesized according to the procedure of Duan *et al*.[36] The bromo-isophosphoramide mustard was synthesized from the corresponding 2-bromoethylamine hydrobromide salt. Then, it was reacted with compound **8** via a Mitsunobu reaction to produce TH-302 with a 51% yield.

For the synthesis of IOS, compound **8** was reacted with TsCl in the presence of DMAP to produce compound **15**. Then, SN-38 was reacted with compound **15** in K_2CO_3/DMF to give

compound **16** (IOS) with an overall yield of 44%. Compound **8** was also treated with bis(4-nitrophenyl)carbonate followed by addition of N-trityl-N,N'-dimethylethylenediamine to produce compound **17**. The trityl protecting group was removed in 10% TFA/DCM to produce compound **18**. Then, SN-38 was treated with bis(4-nitrophenyl)carbonate, followed by addition of the compound **18** solution to produce compound **20** (INS) with an overall yield of 41% (**Scheme 3**).

Scheme 3. Synthesis of IOS and INS



Reagents and conditions: a) TsCl, DMAP, DCM; b)SN-38, K_2CO_3, DMF, DCM; c) bis(4-nitrophenyl)carbonate, N,N'-dimethyl-N-tritylethane-1,2-diamine, DIPEA, DCM; d) 10% TFA/DCM; e) bis(4-nitrophenyl)carbonate, DIPEA, DCM; f) DIPEA, DCM.

2.2. Biological evaluation

2.2.1. In vitro cytochrome P450 metabolism assay

Stability is an important requirement for hypoxia-activated prodrugs of SN-38. Both compounds were stable when treated with PBS (10 mM, pH = 7.4) at 37°C after 48 h. Another critical factor is microsomal stability in the presence of cytochrome P450.[36] As is known, many drugs undergo oxidative and reductive metabolism via cytochrome P450 activation. However, the prodrugs were designed to only be reduced. Thus, we used mouse liver microsomes to assess the metabolism of the prodrugs *in vitro*. When the compounds were treated with mouse liver microsomes for 6 h without NADPH, both compounds were stable. The results indicated that neither of them was a substrate for cytochrome P450 oxidation. Furthermore, treatment of the compounds with mouse liver microsomes

in the presence of NADPH resulted in different stability profiles (**Table 1**). HPLC analysis indicated that IOS was only reduced to SN-38, while INS was converted to other derivatives (**Figure 2**). We hypothesized that IOS underwent a bioreductive metabolism, as we proposed previously. However, INS underwent a more complicated metabolic process, which indicated that the prodrug of SN-38 based on the bis-carbamate linkage could be a substrate of other enzymes besides reductase in cytochrome P450.

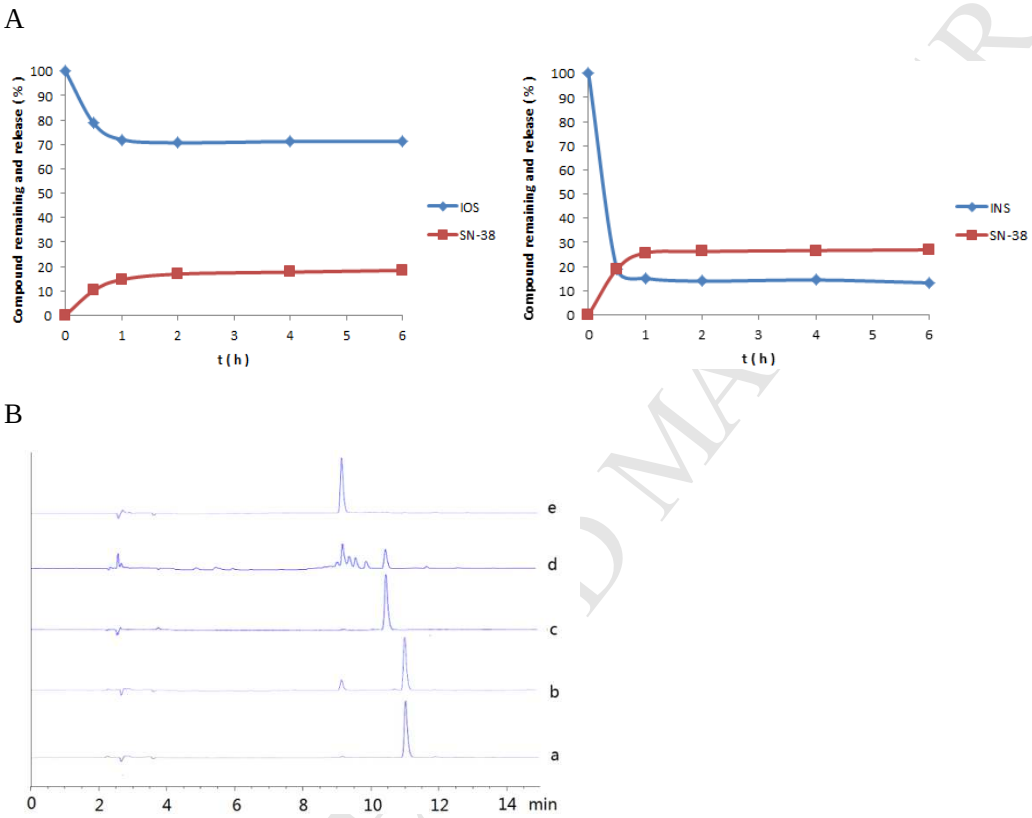


Figure 2. A. Percent of compound remaining and SN-38 releasing in 6h of incubation with mouse liver microsomes in presence of NADPH. B. Compound metabolism with mouse liver microsomes in presence of NADPH detected by HPLC. [a] IOS, t = 0h; [b] IOS, t = 1h; [c] INS, t = 0h; [d] INS, t = 1h; [e] SN-38.

Table 1. In vitro stability of prodrugs

Compound	Percent (%)			
	PBS [a]	MLM [b]	MLM+NADPH [c]	Release of SN-38 [d]
IOS	100	94	72	19
INS	99	92	13	25

[a] Percent of compound remaining after 48h of incubation with PBS. [b,c] Percent of compound remaining after 6h of incubation with mouse liver microsomes in presence or absence of NADPH. [d] Percent of SN-38 releasing after 6h of incubation with mouse liver microsomes in presence of NADPH.

2.2.2. In vitro cytotoxicity assay

The compounds were evaluated for cytotoxicity under normoxic and hypoxic (94% N_2/5% CO_2/1% O_2) conditions using human lung cancer H460 cells and human colon cancer HT29 cells. These cell lines were used for hypoxic/normoxic selectivity tests because they were known to express high levels of DT-diaphorase, an oxygen-insensitive reductase. This widely expressed enzyme has the potential to activate the prodrugs via enzymatic reduction, thereby increasing the toxicity of the prodrugs under normoxic conditions.[36] Cells were treated with the test compounds at various concentrations under a normoxic or hypoxic condition for 24 h and were then washed and incubated under normoxic conditions for up to 72 hours in fresh medium. Cell viability and proliferation were assessed by a MTT assay. The IC_{50} values of the tested compounds for the inhibition of proliferation are shown in **Table 2**.

Table 2. In vitro cytotoxicity assay data summary.

Compound	HT29			H460		
	IC_{50} (μM)			IC_{50} (μM)		
	Hypoxia	Normoxia	HCR[a]	Hypoxia	Normoxia	HCR[a]
TH-302	49.51±7.03	> 100	>2.02	9.08 ± 1.17	50.97 ± 9.90	5.61
SN-38	3.71 ± 0.42	2.74 ± 0.34	0.74	0.06 ± 0.01	0.05 ± 0.01	0.83
IOS	3.89 ± 0.48	14.86 ± 1.69	3.82	0.14 ± 0.03	0.52 ± 0.01	3.71
INS	75.85 ± 6.99	> 100	>1.32	7.56 ± 0.53	8.27 ± 1.22	1.09

[a]Hypoxia cytotoxicity ratio (HCR) was determined by the differential cytotoxicity under normoxic and hypoxic conditions: HCR= IC_{50}(Normoxia)/IC_{50}(Hypoxia).

The results indicated that IOS had moderate hypoxia selectivity and was much more toxic under the hypoxic condition compared with TH-302. The toxicity of IOS was 10-fold higher than that of TH-302. Furthermore, the toxicity of IOS was lower than that of SN-38 under a normoxic condition. However, both hypoxia selectivity and toxicity of INS were lower than those of IOS.

We believe that the different linkages between (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol and SN-38 were responsible for the significant differences in hypoxia selectivity and toxicity of the tested compounds. IOS and INS underwent distinct enzymatic metabolism mediated by cytochrome P450. The results of the assays indicated that IOS was only reduced to SN-38. However, INS containing *N*,*N'*-dimethyl-1,2-ethanediamine may be a substrate of other enzymes besides the reductase in cytochrome P450. INS was converted to other derivatives, which may cause decreased

hypoxia selectivity and toxicity.

3. Conclusions

We developed an improved method for multi-gram scale synthesis of (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol in four steps. This new strategy was used to synthesize compound **8** upon the basic structure of 1-methyl-5-hydroxymethylimidazole. Key steps included the synthesis of 2-azidoimidazole based on the reaction of 2-lithioimidazole with TsN_3 and modified diazotization of 2-aminoimidazole. This procedure was more convenient than the original synthesis method and increased the overall yield from 8% to 42%. The improved method was used for synthesis of TH-302 and hypoxia-activated prodrugs of SN-38. Two different linkages between (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol and SN-38 were evaluated. Compound IOS containing an ether linkage had moderate hypoxia selectivity and more toxicity compared with TH-302. However, INS containing the *N*,*N'*-dimethyl-1,2-ethanediamine may be a substrate of other enzymes besides the reductase in cytochrome P450, which may cause the decreased hypoxia selectivity and toxicity. In conclusion, compound IOS was considered to be a promising hypoxia-selective antitumor agent, and an ether linkage was considered to be effective for the development of more efficient hypoxia-activated prodrugs.

4. Experimental sections

4.1. Materials and methods

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-400 MHz spectrometer (400 and 101 MHz, respectively) using $CDCl_3$, or DMSO-d_6 as solvents with TMS as an internal standard. Chemical shifts were reported as d (ppm) and spin-spin coupling constants as *J* (Hz) values. The mass spectra (MS) were recorded on a Finnigan MAT-95 mass spectrometer. Melting points were taken on a SGW X-4 melting point apparatus, uncorrected and reported in degrees Centigrade. Column chromatography was performed with silica gel (200–300 mesh).

4.2. General synthesis

*2-azido-5-(((tert-butyldimethylsilyl)oxy)methyl)-1-methyl-1H-imidazole (**10**)*

n-Butyllithium (25.5 mL,61.1 mmol, 2.4 N in hexane) was added dropwise to a solution of compound **9** (11.5 g, 50.9 mmol) in anhydrous THF (100 mL) at −78°C. The reaction was stirred at

−78°C for 1 h, then TsN_3 (7.0 g，45.8 mmol) was added dropwise. The reaction mixture was continued stirring at −78°C for 1 h. Then the mixture was quenched with aq. NH_4Cl and extracted with EtOAc. The organic phase was successively washed with brine and H_2O, and dried over Na_2SO_4. Concentration afforded the crude product as yellow oil and was purified by column chromatography on silica gel (heptane–EtOAc 10 : 1) to afford the product **10** (10 g, 82%) as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.73 (s, 1H), 4.55 (s, 2H), 3.40 (s, 3H), 0.87 (s, 9H), 0.04 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.4, 130.3, 125.2, 55.5, 29.6, 25.8, 18.2, -5.3. HRMS (ESI): m/z Calcd for $C_{11}H_{22}N_5OSi$, $[M+H]^+$: 272.1907, found: 272.1829.

*(2-azido-1-methyl-1H-imidazol-5-yl)methanol (**11**)*

Compound **10** (10.0 g, 37.5 mmol) was stirred in 1 N HCl/MeOH (100 mL) at room temperature for 4 h. Then the triethylamine was added to the reaction to adjust pH to 7. The mixture was concentrated in vacuum and then the crude product washed with ether to afford the compound **11** (5.0 g, 87%) as yellow solid. mp 105-107°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.60 (s, 1H), 4.47 (s, 2H), 4.06 (br, 1H), 3.40 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.5, 130.8, 125.1, 54.2, 29.5. MS (ESI) m/z = 154.07 $[M+H]^+$.

*(2-amino-1-methyl-1H-imidazol-5-yl)methanol (**12**)*

Compound **11** (5.0g, 32.7mmol) was dissolved in 50 mL of MeOH, then Pd/C (0.5g of 10%Pd) was added and the mixture was hydrogenated under hydrogen balloon for 1 h. The catalyst was removed by filtration and the filter was evaporated by vacuum to afford compound **12** (4.0 g, 96%) as gray solid. mp 177-179°C. ^{1}H NMR (400 MHz, DMSO) δ 6.25 (s, 1H), 5.26 (br, 2H), 4.78 (br, 1H), 4.25 (s, 2H), 3.28 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 150.1, 126.5, 122.2, 53.2, 28.7. HRMS (ESI): m/z Calcd for $C_5H_{10}N_3O$, $[M+H]^+$: 128.0824, found: 128.0819.

(1-methyl-2-nitro-1H-imidazol-5-yl)methanol (8)

Compound **12** (4.0 g, 31.5 mmol) was dissolved in fluoboric acid (40% w/v, 20 mL). The solution was cooled to -15°C, and a solution of sodium nitrite (3.3 g, 47.3 mmol) in water (10 mL) was added dropwise. The solution was stirred at -15°C for 30 min, then added dropwise into a solution of Cu powder (2.0 g, 119 mmol) and sodium nitrite (21.7 g, 157.5 mmol) in water (50 mL).The reaction

mixture was stirred at room temperature for 1 h. The solution was extracted with EtOAc. The organic extract was evaporated in vacuum, and the residue was eluted by DCM to afford compound **8** (3.0 g, 61%) as yellow solid. mp 142-144°C. ^{1}H NMR (400 MHz, DMSO) δ 7.11 (s, 1H), 5.47 (t, *J* = 5.4 Hz, 1H), 4.54 (d, *J* = 5.4Hz, 2H), 3.92 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 145.7, 138.7, 126.6, 53.0, 34.1. HRMS (ESI): m/z Calcd for $C_5H_8N_3O_3$, $[M+H]^+$: 158.0487, found: 158.0560.

(1-methyl-2-nitro-1H-imidazol-5-yl)-N,N–bis(2-bromoethyl) phosphordiamidate (TH-302)

To a suspension of N,N'-bis(2-bromoethyl)phosphorodiamidic acid (50 mg, 0.16 mmol), 1-methyl-2-nitroimidazole-5-methanol (50 mg, 0.32 mmol), and PPh_3 (84 mg, 0.32 mmol) in THF (15 mL) was added DIAD (0.13 mL, 0.32 mmol) at 0°C. After the addition of DIAD, the reaction mixture was warmed to room temperature and stirred overnight. The solvent was removed, and the residue was purified by column chromatography on silica gel (DCM : MeOH = 50:1) to afford compound **14** (37 mg, 51%) as yellow gum. ^{1}H NMR (400 MHz, DMSO) δ 7.25 (s, 1H), 5.05-4.99 (m, 2H), 4.98 (d, *J* = 7.6 Hz, 2H), 3.94 (s, 3H), 3.42 (t, *J* = 6.9 Hz, 4H), 3.17-3.05 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 146.1, 134.1(d, *J* = 8.0 Hz), 128.2, 55.7 (d, *J* = 4.3 Hz), 42.7, 34.3, 34.1 (d, *J* = 5.0 Hz). HRMS (ESI): m/z Calcd for $C_9H_{17}Br_2N_5O_4P$, $[M+H]^+$: 469.9204, found: 469.9186.

*5-(chloromethyl)-1-methyl-2-nitro-1H-imidazole (**15**)*

To a stirred solution of compound **8** (47 mg, 0.3 mmol) and DMAP (73 mg,0.6 mmol) in DCM (3 mL) was added TsCl (69 mg, 0.36 mmol). The reaction was stirred for 4 h at room temperature. The mixture was concentrated in vacuum and purified by column chromatography on silica gel (heptane : EtOAc = 10 : 1) to afford compound **15** (50mg, 95%) as yellow solid. mp 96-98°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (s, 1H), 4.62 (s, 2H), 4.07 (s, 3H). MS (ESI) m/z = 175.87 $[M+H]^+$.

*Compound **16** (IOS)*

To a stirred solution of SN-38 (78 mg, 0.2 mmol) and K_2CO_3 (27 mg, 0.2 mmol) in anhydrous DMF (2 mL) and DCM (2 mL) was added compound **15** (35 mg, 0.2 mmol). The mixture was stirred overnight under nitrogen balloon at room temperature. The reaction was quenched with H_2O and extracted with EtOAc. The combined organic layers were dried over Na_2SO_4. Concentration was purified by column chromatography on silica gel (DCM : MeOH = 50 : 1) to afford compound **16**

(50 mg, 47%) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, *J* = 9.2 Hz, 1H), 7.62 (s, 1H), 7.54 – 7.44 (m, 2H), 7.33 (s, 1H), 5.75 (d, *J* = 16.2 Hz, 1H), 5.31 (d, *J* = 16.3 Hz, 1H), 5.27 (s, 2H), 5.26 (s, 2H), 4.15 (s, 3H), 3.75 (s, 1H), 3.17 (q, *J* = 7.5 Hz, 2H), 1.98 – 1.82 (m, 2H), 1.42 (t, *J* = 7.6 Hz, 3H), 1.04 (t, *J* = 7.4 Hz, 3H). HRMS (ESI): m/z Calcd for $C_{27}H_{26}N_5O_7$ ($M+H^+$): 532.1832, found: 532.1815.

*(1-methyl-2-nitro-1H-imidazol-5-yl)methyl methyl(2-(methyl(trityl)amino)ethyl)carbamate (**17**)*

To a stirred solution of compound **8** (30 mg, 0.19 mmol) and DIPEA (49 mg, 0.38 mmol) in DCM (5 mL) was added bis(4-nitrophenyl)carbonate (64 mg, 0.21 mmol). The reaction was stirred for 4 h at room temperature, then was added N,N'-dimethyl-N-tritylethane-1,2-diamine (94 mg, 0.29 mmol). The mixture was stirred for another 1h. Then the solution was washed with Na_2CO_3 and extracted with DCM. The combined organic layers were dried over Na_2SO_4. Concentration was purified by column chromatography on silica gel (heptane : EtOAc = 2 : 1) to afford compound **17** (88 mg, 91%) as yellow solid. mp 100-102°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 – 7.37 (m, 6H), 7.26 – 7.19 (m, 6H), 7.18 – 7.11 (m, 3H), 7.08 (s, 1H), 5.19 – 5.01 (m, 2H), 4.05 – 3.62 (m, 3H), 3.56 – 3.37 (m, 2H), 3.13 – 2.89 (m, 3H), 2.38 – 2.18 (m, 2H), 2.18 – 2.05 (m, 3H). MS (ESI) m/z = 514.37 $[M+H]^+$.

*Compound **20** (INS)*

To a stirred solution of compound **17** (40 mg, 0.078 mmol) in DCM (2 mL) was added trifluoroacetic acid (0.2 mL). The reaction was stirred for 1 h at room temperature, and concentrated in vacuum to afford the crude product of compound **18**. In another bottle, bis(4-nitrophenyl)carbonate (24 mg, 0.078 mmol) was added into the DCM solution (5 mL) of SN-38 (31 mg, 0.078 mmol) and DIPEA (20 mg, 0.156 mmol), which afforded the compound **19** after 3 h reaction. Then compound **18** was added into the solution of compound **19** without purification. Appropriate DIPEA was added to keep pH among 7~8. The reaction was stirred at room temperature for 3 h. The mixture was concentrated in vacuum and purified by column chromatography on silica gel (DCM : MeOH = 50 : 1) to afford compound **20** (24 mg, 45%) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 – 8.18 (m, 1H), 7.90 – 7.77 (m, 1H), 7.66 (s, 1H), 7.60 – 7.45 (m, 1H), 7.24 – 7.13 (m, 1H), 5.78 – 5.68 (m, 1H), 5.32 – 5.15 (m, 5H), 4.09 – 3.91 (m, 4H), 3.75 – 3.43 (m, 4H), 3.24 – 2.93 (m, 8H), 1.98 – 1.81 (m, 2H), 1.46 – 1.32 (m, 3H), 1.02 (t, *J* =

7.3 Hz, 3H). HRMS (ESI): m/z Calcd for $C_{33}H_{36}N_7O_{10}$ $[M+H]^+$: 690.2524, found: 690.2515.

4.3. General procedure for Cytochrome P450 reductase assay

Mouse liver microsomes (20 mg/mL) were purchased from Corning. NADPH was purchased from Sigma-Aldrich.

The prodrugs measurements were made in 10 mM PBS (pH 7.4) according to the following procedure. 1 μL of 5 mM prodrug in DMSO were diluted in 1mL PBS, followed by addition of $MgCl_2$ (5 μmol), NADPH (1 μmol), and 50 μL mouse liver microsomes. The incubation mixtures of test compounds with mouse liver microsomes contained the following at the indicated final concentrations: 10 mM PBS (pH 7.4), 5 μM test compound, 5 mM $MgCl_2$, microsomes (1 mg/mL) with or without 1 mM NADPH. The mixtures were preincubated at 37 °C. The metabolism reaction was initiated by the addition of 50 μL mouse liver microsomes solution. At 0, 0.5, 1, 2, 4, 6 h, 100uL reaction mixture was quenched by 100 μL cold methanol. The samples were centrifuged at 4 °C for 5 min at 12000 rpm. The supernatant was transferred to a vial for analysis using HPLC.

4.4. In Vitro Cell-Proliferation Assay

Human lung cancer cell line H460 and human colon cancer cell line HT29 were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China).

H460 cells and HT29 cells were cultured in RPMI 1640 medium and McCoy's 5A medium respectively, supplemented with 10% fetal bovine serum and 1% penicillin streptomycin (Invitrogen) in a 5%CO_2 humidified environment at 37 °C. Cells were seeded in 96-wellplates and incubated for 24 h. The next day, serial dilutions of test compounds in DMSO were made in RPMI 1640 medium or McCoy's 5A medium, such that final concentrations of DMSO did not exceed 0.1%. The cells in the hypoxia treatment group were incubated for 24h in the anaerobic chamber flushed with a certified anaerobic gas mixture (94% N_2/5% CO_2/1% O_2; Thermo). The cells in the air treatment group were incubated for 24 h in standard cell-culture incubator. After the 24 h of treatment with test compounds, the cells were washed with 200 μL PBS and incubated up to 72 h in 200 μL of fresh medium. After 3 days, the cells were treated with MTT and measured by a SpectraMax M5 microplate reader at an emission wavelength of 570 nm. The 50% inhibitory concentration (IC_{50}) of test compounds was calculated from results (n=3).

4.5. General HPLC Method

HPLC analysis was performed at room temperature using a Diamonsil C_{18} (250 mm × 4.6 mm) and a mobile phase gradient from 5% CH_3CN/buffer (0.1% TFA/H_2O) to 95% CH_3CN /buffer (0.1% TFA/H_2O) for 15 min, a flow rate of 1.0 mL/min, and plotted at 373 nm. This method was used to determine the purity for the tested compounds, and also used in stability studies.

Supplementary data

Supplementary data related to this article can be found at.

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Figure 1. Proposed mechanism of prodrugs activated via enzymatic metabolism under hypoxic conditions.

Figure 2. A. Percent of compound remaining and SN-38 releasing in 6h of incubation with mouse liver microsomes in presence of NADPH. B. Compound metabalism with mouse liver microsomes in

presence of NADPH detected by HPLC. [a] IOS, t = 0h; [b] IOS, t = 1h; [c] INS, t = 0h; [d] INS, t = 1h; [e] SN-38.

Table 1. In Vitro Stability of Prodrugs. [a] Percent of compound remaining after 48h of incubation with PBS. [b,c] Percent of compound remaining after 6h of incubation with mouse liver microsomes in presence or absence of NADPH. [d] Percent of SN-38 releasing after 6h of incubation with mouse liver microsomes in presence of NADPH.

Table 2. In vitro cytotoxicity assay data summary. [a]Hypoxia cytotoxicity ratio (HCR) was determined by the differential cytotoxicity under normoxic and hypoxic conditions: HCR= IC_{50}(Normoxia)/IC_{50}(Hypoxia).

Scheme 1. Original synthesis. **Reagents and conditions: A.** a) K_2CO_3, HCOOEt, EtOH; b) NaH, HCOOEt; c) HCl, EtOH; d) NH_2CN, AcONa, H_2O, 15% over four steps; e) AcOH, $NaNO_2$, 62%;f) NaOH, H_2O, 95%; g) isobutyl chloroformate, $NaBH_4$, THF, 88%. **B.** h) HCOOEt, THF, NaH; i) EtOH, conc. HCl; j) EtOH, H_2O, NH_2CN, 48–54% over three steps.

Scheme 2 New synthesis. **Reagents and conditions:** a) n-BuLi, i-PrONO_2, THF; b) n-BuLi, TsN_3, THF, 82%; c) HCl, MeOH, 4h, 87%; d) Pd/C, H_2, MeOH, 1h, 96%; e) $NaNO_2$, Cu, HBF_4, H_2O, 61%.

Scheme 3. Synthesis of IOS and INS. **Reagents and conditions:** a) TsCl, DMAP, DCM; b)SN-38, K_2CO_3, DMF, DCM; c) bis(4-nitrophenyl)carbonate, N,N'-dimethyl-N-tritylethane-1,2-diamine, DIPEA, DCM; d) 10% TFA/DCM; e) bis(4-nitrophenyl)carbonate, DIPEA, DCM; f) DIPEA, DCM.

Highlights

- We proposed new hypoxia-activated prodrugs that conjugated (1-methyl-2-nitro-1H-imidazol-5-yl)methanol with SN-38.
- We improved the synthesis of (1-methyl-2-nitro-1H-imidazol-5-yl)methanol, which increased the yield from 8% to 42%.
- Compound IOS was evaluated to be a promising hypoxia-selective antitumor agent.