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Highly GSH-sensitive SN-38 prodrug with “OFF-to-ON” fluorescence switch as a bifunctional anticancer agent

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Abstract

SN-38 (7-ethyl-10-hydroxy-camptothecin) is an active metabolite of irinotecan (CPT-11) and the most potent camptothecin analogue. In this study, 2,4-dinitrobenzene sulfonyl (DNS) was covalently conjugated as GSH-sensitive trigger to 10' -OH of SN-38 to yield a GSH-sensitive prodrug, denoted as DNS-SN38, with virtually quenched fluorescence due to donor-excited photo-induced electron transfer (d-PeT). By investigating DNS-SN38's activation property upon fluorescence restoration and cytotoxic potency against ovarian cancer cell lines (A2780 and m-Cherry+OCSC1-F2), its potential applicability as a useful chemotherapeutic agent was demonstrated.

Since its conception, the prodrug strategy has gained a considerable interest in the industry for its potential to enhance the pharmaceutical (PC), pharmacokinetic (PK), and/or pharmacodynamic (PD) properties of the chemotherapeutic agents, and simultaneously circumvent the significant side effects presented by conventional chemotherapy.¹ Particularly for cancer chemotherapy, the prodrug systems that have been engineered with stimuli-sensitive activation were recognized for their enhanced therapeutic efficiency as well as their drug release specificity.

The tumor microenvironment (TME) is known to encompass unique physicochemical properties that are significantly different from those of the normal tissues. One of the main highlights of TME is generally accepted to be the elevated oxidative stress resulting from the overproduction of reactive oxygen species (ROS) by hypoxic conditions in various TME-associated cells.² Although normal physiological levels of ROS are essential for cell survival, excess formation of ROS elicits permanent cell damage and death. Thus, the ROS overexpression in TME concomitantly upregulates antioxidant systems in order to achieve

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redox homeostasis.^{2(b)} Glutathione (GSH), an endogenous tripeptide composed of glutamate, cysteine, and glycine, is recognized to be the major antioxidant agent that participates in the maintenance of such redox imbalance by directly scavenging free radicals and peroxides.³ Furthermore, GSH's contribution towards cancer cell proliferation has been prominently implicated in a number of previous studies, where GSH was found to play crucial roles in various cellular processes, such as detoxification of xenobiotics and cell cycle progression. In fact, elevated GSH levels have been detected in various types of human cancer tissues.^{3(a),4} In light of this, exploiting elevated GSH levels for stimuli-sensitive activation is a trending paradigm in designing drug delivery systems for cancer chemotherapy.⁵

SN-38 (7-ethyl-10-hydroxy-camptothecin), an active metabolite of irinotecan (CPT-11), is considered to be the most potent antineoplastic agent among the camptothecin (CPT) derivatives. The CPT analogues are known to act as inhibitors of topoisomerase-1 (Top-1), which is responsible for relieving the torsional strain in DNA.⁶ The therapeutic efficacy of CPT-11 is directly related to its metabolic conversion into SN-38 by carboxylesterases 1 and 2, as SN-38 exhibits potency of up to 1000-fold in comparison to that of CPT-11. However, due to a relatively low conversion rate (<10%) and significant interpatient discrepancies in carboxylesterase efficacy,⁷ a prodrug design that can achieve an enhanced and consistent SN-38 conversion efficiency is considered vital in fully exploiting its cytotoxic potency.

Herein, we report the successful synthesis of a bifunctional SN-38 prodrug, denoted as DNS-SN38, that is highly sensitive towards the GSH-triggered activation. In this prodrug, 2,4-dinitrobenzene sulfonyl (DNS) is directly conjugated to SN-38 as a cleavable trigger via nucleophilic addition of the sulfhydryl (R-SH) group in the endogenous reducing agents, such as GSH and cysteine (Cys). Recently, the DNS group has emerged as a notable trigger in designing thiol-sensitive prodrugs and fluorescent imaging probes, where its strong electron-withdrawing potential allowed the quenching of intrinsic fluorescence of the conjugated fluorophores via donor-excited photo-induced electron transfer (d-PeT).⁸ The DNS moiety allowed the transfer-acceptance of the excited electron from the fluorophore and thereby distorted the normal emission mechanisms. As SN-38 is one of the chemotherapeutic agents that exhibits intrinsic fluorescence, we believed engineering SN-38 with this fluorescence quenching mechanism may suggest future directions in utilizing the drug as a molecular diagnostic imaging agent for facile tracking of drug distribution. Furthermore, the covalent masking of SN-38's C₁₀ hydroxyl (-OH) with the DNS may sufficiently attenuate the drug's Top-1 inhibiting activity until unmasking, as the respective site contributes towards the stability of SN-38-induced Top-1-cleavable complexes.⁹ Considering GSH concentration is found to be dominant in the intracellular regions and especially elevated in hypoxic tumors,^{3(a),4} DNS-SN38 has the potential to become a viable bifunctional anticancer prodrug that enables both its therapeutic and diagnostic functions at the site of action when it is sufficiently supplemented with nanotechnology-driven delivery platform(s) in the future. For these reasons, we have carried out a preliminary investigation on DNS-SN38 and demonstrated its therapeutic scope for advanced chemotherapy.

The synthetic route for DNS-SN38 is depicted in Scheme S1 (†ESI). By precisely controlling the reagent ratio, temperature, and addition rate, selective conjugation of DNS to

the phenolic hydroxyl group (C₁₀-OH) was accomplished. In addition to ¹H nuclear magnetic resonance (NMR) (Fig. S1, [†]ESI) validation of the prodrug structure, we utilized high performance liquid chromatography (HPLC) to confirm the success of the respective modification. Based on the detection conditions listed in details in [†]ESI, the retention times for SN-38 and DNS-SN38 were determined to be 2.1-minutes and 7.1-minutes, respectively. Considering the chromatography was performed in reversed-phase, this result indicated that DNS-SN38 was more hydrophobic than SN-38. Such increase in hydrophobicity may be attributed to the masking of polar hydroxyl group with highly non-polar DNS moiety. Previously, SN-38 was modified with various hydrophobic groups, such as oleic acid,¹⁰ valine,¹¹ and vitamin E¹² to increase the lipid solubility of the drug. The potential benefits of such modifications were: enhanced drug loading efficiency in lipid-based formulations and improved permeability through the cell membranes. Although DNS may not be on par with the aforementioned groups in hydrophobic contribution, its presence may still be beneficial for the preparation of self-assembly-driven nanomedicine system for future studies. For example, a covalent modification of a hydrophobic functional group with polyethylene glycol (PEG) – “PEGylation” – to formulate a self-assembled nanoparticle system has become a common approach for creating an effective delivery platform with increased blood circulation time and reduced systemic clearance.¹³ For DNS-SN38, such modification could yield significant enhancements in its PK properties.

The large variation in cytotoxic potency between the CPT analogues mainly derives from the differences in arrangements and/or substitutions in their chemical structures.⁷ In fact, the presence of a chiral center at C₂₀ creates 20*S*- and 20*R*-isomers of CPT, where the *S*-form exhibits potency of up to 100-fold relative to its counterpart.^{7,14} In the case of SN-38, it has been reported that the C₁₀-OH and C₇ ethyl groups contribute toward the stability of Top-1 cleavable complexes induced by SN-38 and subsequently contribute largely to its unparalleled cytotoxicity.^{9(c)} As such, covalent masking of C₁₀-OH has proven to significantly lower the cytotoxicity of SN-38 as exemplified by several cases, such as CPT-11 and SN38-glucuronide (SN38G).¹⁵ Thus, we rationally anticipate DNS-SN38 to sufficiently follow the most fundamental principle of prodrugs – pharmacological inertness prior to activation.

In order to test our hypothesis on the GSH-triggered activation of DNS-SN38, 2 μM of DNS-SN38 was first treated with 1 mM of GSH in DMSO:PBS (10 mM, pH 7.4) solution at 37°C for 10 minutes and analyzed via HPLC. In the resulting chromatogram (Fig. 1 (A)), the characteristic peak of DNS-SN38 at 7.1-minutes was fully converted into a new peak at 2.1-minutes, which was previously characterized to represent the SN-38 standard. As the control group (without GSH-treatment) remained unchanged in the same incubation conditions, this result indicated that GSH was able to cleave off the DNS moiety and thereby release intact SN-38.

Encouraged by this result, DNS-SN38's activation kinetics was also studied in terms of its fluorescence restoration. As mentioned previously, we predicted that the covalent conjugation of DNS to SN-38 may quench the drug's intrinsic fluorescence at 556 nm due to

[†]Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

d-PeT process. Therefore, we first examined the optical properties of DNS-SN38 in comparison to those of SN-38. The UV-Vis absorption spectroscopy of DNS-SN38 revealed that the absorption maxima were present at 365 nm and 380 nm (Fig. S2, [†]ESI). Since SN-38's maximum absorption band was also present at 365 nm, the excitation wavelength ($\lambda_{\text{excitation}}$) was set accordingly for the fluorescence study.

Parallel with our anticipation, Fig. 1(B) depicts the fluorescence emission of 20 μM of DNS-SN38 (DMSO:PBS) to be virtually quenched at 556 nm, while the emission of 20 μM of SN-38 is shown to be 41-times higher in intensity than that of DNS-SN38. In order to verify that this quenched fluorescence is restorable, the DNS-SN38 sample was then treated with 100 μM of GSH in the same conditions and its fluorescence emission was monitored in a time-dependent manner. As Fig. 1(C) displays, the GSH-triggered activation of DNS-SN38's fluorescence was completed within 9.5-minutes of the treatment, which was consistent with the activation rate observed in the preliminary study carried out using HPLC. However, considering the molar ratio of [GSH:DNS-SN38] for the fluorescence study, [10:2], was significantly lower than the HPLC study, [1000:2], we could deduce that the GSH-triggered DNS-cleavage occurs very efficiently to induce a favorable prodrug activation rate.

In order to further assess such efficient prodrug activation, the fluorescence restoration of DNS-SN38 was monitored in a GSH-dependent manner in similarly prepared conditions. As shown in Fig. 1(D), an array of GSH concentrations (0, 20, 40, 60, and 100 μM) was tested against 20 μM of DNS-SN38. The results revealed that the prodrug's activation was indeed dependent on the concentration of GSH as notable increases in the activation rate were detected with increases in GSH concentration. Furthermore, the rapid activation trend observed in our previous studies remained consistent as all tested samples reached their plateau intensities within 10-minutes, further demonstrating the high activation efficiency of DNS-SN38. It should be noted that the 20 μM GSH-treated sample, [1:1], displayed a plateau intensity of around 82% of the maximum plateau intensity detected in the remaining samples with higher GSH contents. This indicated that a slight molar excess of GSH, over DNS-SN38, is still required to induce a complete activation of the prodrug. Finally, considering that the control group without GSH-treatment remained quenched in its fluorescence emission in the same incubation conditions for hours, it was explicitly confirmed that the DNS-SN38 activation was triggered by GSH.

Up-to-date, the most commonly used trigger for GSH-sensitivity in various prodrug systems has been the disulfide bond (R-SS-R'). While various disulfide linkers have provided several advantages, such as the capability to serve as a structural linker between the two functional moieties, their thiol-triggered self-immolation showed relatively delayed responses and required significant excess of thiols for activation. For example, a disulfide-bridged prodrug system consisting of a naphthalimide derivative (NAP) and CPT required 180 minutes for a complete activation in [1000:2] (GSH:NAP-SS-CPT) condition.^{5(b)} Moreover, for the activation of similarly designed CPT-SS-CyN (cyanine-amine) system, 120 minutes was required in 100-fold excess of dithiothreitol.¹⁷ Compared to these previous reports, it was apparent that the GSH-triggered DNS-cleavage was superior in terms of the activation efficiency.

Next, confocal microscopy was utilized to examine the real-time drug tracking capability of DNS-SN38. Here, the nuclei of B16F10 cells were stained with DAPI (4',6-diamidino-2-phenylindole) and subsequently incubated with 10 μ M of DNS-SN38 for analysis. As the images in Fig. 2 show, DNS-SN38 initially displayed minimal green fluorescence at the 1-minute mark, indicating that the drug internalization has not yet occurred and its quenched fluorescence was intact. However, starting from the 5-minute mark, a noticeable increase in fluorescence emission was observed and the image eventually became saturated with green fluorescence by the 30-minute mark. This trend clearly indicated that the DNS-SN38 activation could be achieved rapidly as a result of cellular internalization of the drug. The fluorescence intensities of DNS-SN38 detected at different time-points were then normalized to the DAPI fluorescence intensity to quantify the respective trend (Fig. S3, [†]ESI), where around 80% of the normalized intensity was detected at the 30-minute mark. To further assess the drug internalization, the images were then merged with DAPI fluorescence images. The expanded view of the merged images in Fig. 2 indicated nuclear localization of DNS-SN38 as the blue fluorescence representing B16F10 cell nuclei were surrounded by the green fluorescence of the activated DNS-SN38.

The results of our studies collectively showed that the GSH-triggered activation of DNS-SN38 is highly efficient and rapid. Although the results obtained from such simulated '*in vitro*' conditions cannot be used as the sole evidence to envision the trends in complex physiological conditions, the results still reasonably suggested that the activation of our prodrug within the intracellular regions – GSH in millimolar (mM) range – is extremely probable.

Finally, the anticancer efficacy of DNS-SN38 was evaluated in comparison to SN-38 against A2780 cell and mCherry+OCSC1-F2 (mCherry-labeled ovarian cancer stem cell) lines. In Fig 3., the 24-hour cell viability profiles for both cell lines are displayed where DNS-SN38 induced nearly identical profiles to those of SN-38. This behavior further indicated that DNS-SN38's conversion into SN-38 is highly efficient. Furthermore, the IC₅₀ (50% inhibitory concentration) values were determined to be within 10 nM for both DNS-SN38 and SN-38 against both cell lines. Considering some CPT analogues typically display IC₅₀ in micro-molar range, it was apparent that the cytotoxicity of activated DNS-SN38 is superior and comparable to that of the intact SN-38.

In summary, we have reported the successful synthesis and preliminary characterizations of a bifunctional SN-38 prodrug, DNS-SN38, that is highly sensitive towards the GSH-triggered activation and displays quenched fluorescence. Through various studies, we have demonstrated DNS-SN38's highly efficient prodrug activation, capability for real-time monitoring of drug distribution, and potent cytotoxicity that was comparable to that of SN-38. Thus, we propose DNS-SN38 as a stimuli-sensitive prodrug agent that has the potential to be translated into a useful chemotherapeutic option.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) HPLC chromatograms of DNS-SN38, GSH-treated (10 minute) DNS-SN38, and SN-38 standard. (B) Fluorescence emission at 556 nm ($\lambda_{\text{excitation}} = 365$ nm) of SN-38 standard and quenched emission of DNS-SN38. (C) Fluorescence activation of DNS-SN38 with 100 μ M GSH over 9.5 minutes with consistent 60-second intervals. (D) GSH concentration (control or 0, 20, 40, 60, 100 μ M)-dependent fluorescence activation rates of DNS-SN38 (20 μ M). The error bars represent standard deviation ($n=3$).

Figure 2. Confocal microscopy images of B16F10 cells incubated with 10 μ M of DNS-SN38 at different time-points (1, 5, 15, and 30 minutes) for fluorescence intensity analysis. The nuclei were stained with DAPI (blue). The green fluorescence shows the location of activated DNS-SN38, where the expanded images represent the regions of interest (ROI) in yellow boxes. Scale bars of DAPI, DNS-SN38, and merged images represent 100 μ m. Scale bars of the expanded images represent 20 μ m.

Figure 3.

(A) 24-hour cell viability profiles for A2780 cell line treated with either DNS-SN38 or SN-38 at varying concentrations (nM); (B) 24-hour cell viability profiles for mCherry +OCSC1-F2 cell line treated with either DNS-SN38 or SN-38 at varying concentrations (nM). The error bars represent standard deviation ($n=6$).

Scheme 1.

Schematic illustration of DNS-SN38 fluorescence activation in the presence of biothiols.

The quenched fluorescence of SN-38 is resumed upon DNS-cleavage by thiol substitution.