Bioorganic & Medicinal Chemistry Letters 19 (2009) 5016–5020

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Human plasma-mediated hypoxic activation of indolequinone-based naloxone pro-drugs

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ARTICLE INFO

Article history:
Received 14 May 2009
Revised 7 July 2009
Accepted 9 July 2009
Available online 12 July 2009

Keywords:
Hypoxia
Selective drug release
Indolequinone
Naloxone
Human plasma

ABSTRACT

Hypoxia is known to occur in tissues in response to narcotic analgesic therapy using as a result of respiratory depression. The aim of this study was to synthesize a narcotic antagonist pro-drug that can be activated by tissue hypoxia to prevent the damage associated with respiratory depression. We synthesized three different pro-drugs of the narcotic antagonist naloxone utilizing indolequinone as the hypoxia-sensitive moiety. The indolequinone structure in the pro-drugs was designed to have an open reactive point at the N-1 position offering the possibility of further conjugation with macromolecules to modify the bioavailability of these pro-drugs in vivo. A pro-drug (labeled **1**) where naloxone and the indolequinone moiety were linked through a carbonate bond was rapidly hydrolyzed in phosphate buffered saline. However, two additional pro-drugs (labeled **2** and **3**) having carbamate linkers were stable in phosphate buffered saline for 24 h. The reductive release of naloxone from the pro-drugs was achieved in the presence of the bio-reductive enzyme DT-Diaphorase, with about 80% release occurring from the two pro-drugs in 24 h. More than 99% of naloxone was released from pro-drug **2** in 30% human plasma, however the release only occurred under hypoxic conditions. This system provides a potential means for feedback control to counter critical respiratory depression induced by narcotic analgesics.

There has been continued interest in the exploitation of indolequinones as a method of hypoxia-targeted drug delivery.[1,2] Hypoxic cells and tissues have been documented to occur in cardiovascular diseases,[3] wound healing,[4] and neurodegenerative,[5] inflammatory[6] and gastrointestinal disorders.[7] Hypoxic cells are generally characterized by low oxygen tension, low pH, low nutrient levels, and over-expression of angiogenic factors leading to cell death and permanent tissue damage. This has led to the development of bioreductive indolequinone-based pro-drugs that can potentially target hypoxic cells.

Narcotic analgesics are the most effective pain relief medications for acute traumatic injury. However, narcotics have serious side effects, such as hypoxia due to respiratory depression and hypotension due to vasodilation.[8] These adverse effects may cause cardio-respiratory collapse in the setting of traumatic injury, potentially leading to death. One approach to counteract adverse narcotic effects would be to use low-level narcotic antagonist feedback that prevents respiratory failure while maintaining analgesia. Applying an agonist-antagonist based strategy would involve a combination therapeutic containing both a narcotic agonist to achieve analgesia and a narcotic antagonist whose release could be activated in the presence of tissue hypoxia to reverse respiratory depression. Specifically, the feedback component would need to rapidly release a short-acting narcotic antagonist when a low oxygen pressure is present. Naloxone is an effective, short-acting opioid antagonist that can reverse a range of morphine's effects, including analgesia and respiratory depression.[9] Although morphine's analgesic and respiratory effects overlap, low-dose infusions of naloxone can reverse morphine's respiratory depression, while its analgesic effect can be relatively unaffected allowing pain relief to continue.[10] We report here the synthesis and reductively-triggered drug release studies of naloxone pro-drugs bearing an indolequinone structure.

The structures of indolequinone bearing naloxone pro-drugs **1**, **2**, and **3** are shown in Scheme 3. The relationship between pro-drug structures and drug release kinetics has been previously studied.[1] Generally, compounds with electron-withdrawing groups at the indole 3-position were among the best substrates, and that groups larger than methyl at N-1 position are clearly tolerated by DT Diaphorase enzyme for bioreduction. A study on the reductively triggered release of a variety of oxygen-based and sulfur-based exiting groups from their 4,7-dioxoindole-3-methyl derivatives was carried out.[11] It has also been demonstrated that upon substitution with a potential leaving group at the 3-position of the indolequinone moiety is a viable means for bioreductive drug delivery.[12]

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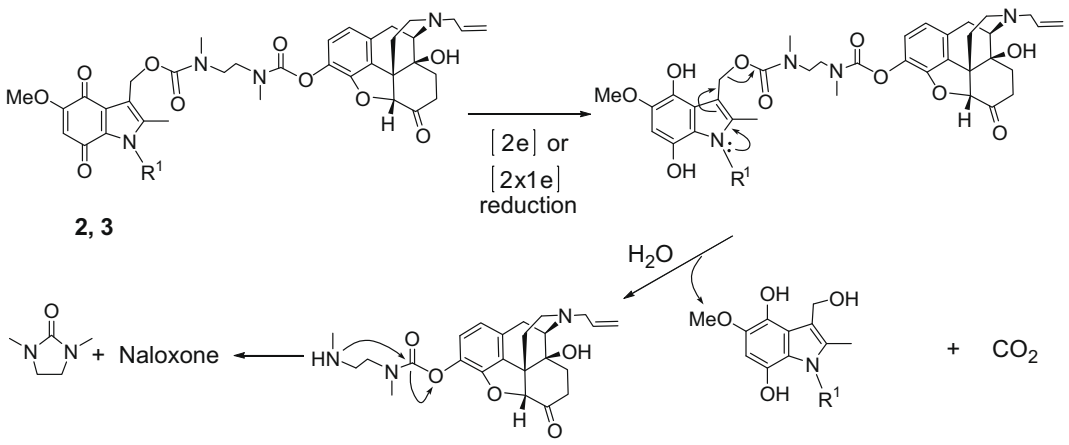
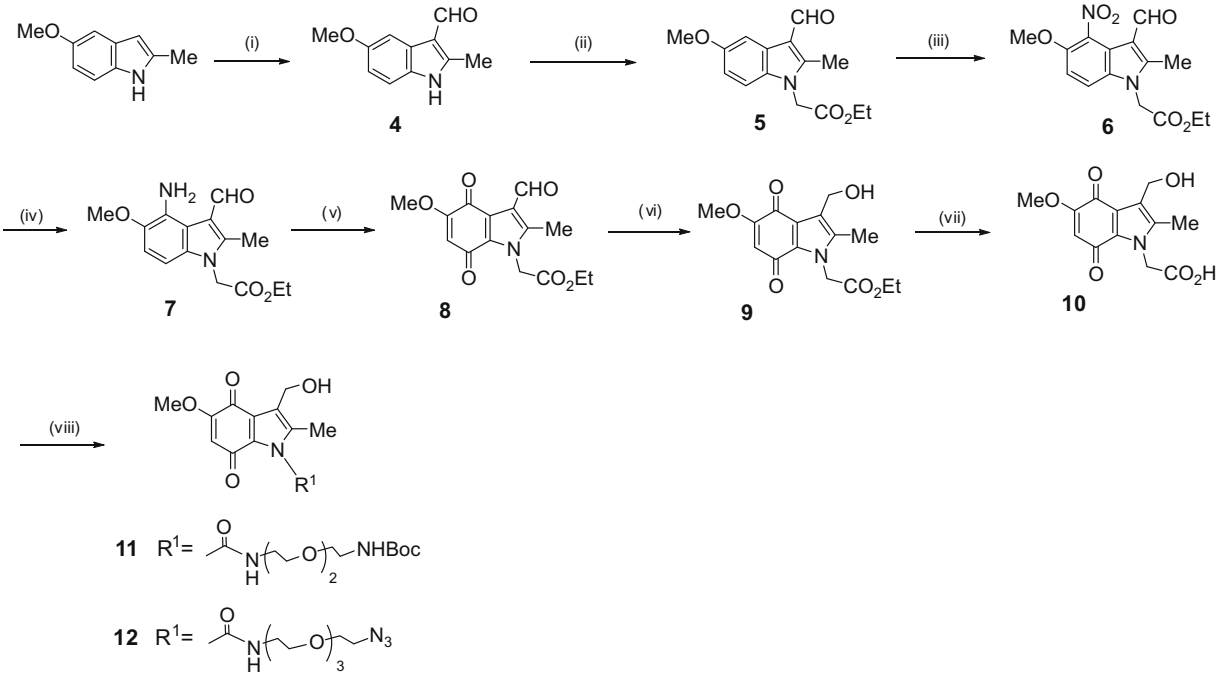
doi:10.1016/j.bmcl.2009.07.061



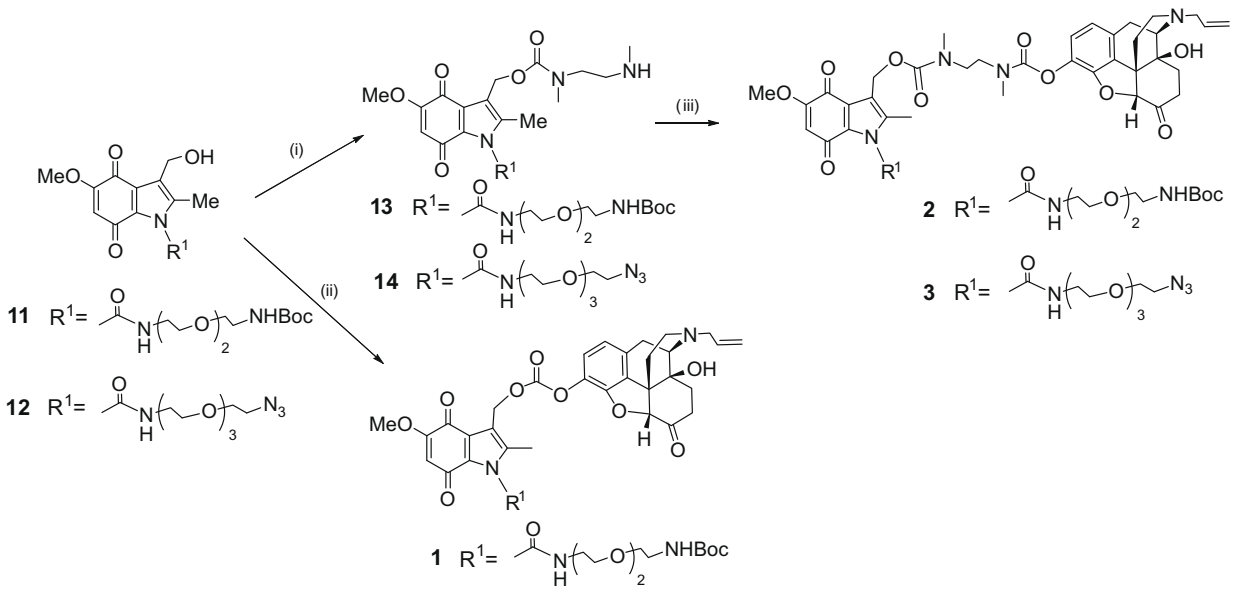
Scheme 1. Proposed mechanism of reductively triggered release of naloxone from pro-drugs **2** and **3**.

In the current study, we used the indolequinone structure with 2-methyl 5-methoxy groups that has shown good drug release properties. A proposed chemical structure for reductively activated release of naloxone is shown in Scheme 1. When the pro-drugs **2**, and **3** are reduced to dihydroxyindole, the electron density at the indole nitrogen increases drastically, triggering expulsion of the exiting group (in this case, naloxone), in a reverse-Michael-like process, releasing the drug coupled to a small spacer.[13,14]

The synthesis of the indolequinone linker compounds **11** and **12** is demonstrated in Scheme 2. The methyl analogue of the indolequinone structure has been synthesized by Naylor et al.[11] Rather than putting a methyl group at the N-1 position, we used an ethoxycarbonyl methyl functional group at the N-1 position. This synthetic design allows this position to serve as an open reaction site to facilitate conjugation of long chain tethers or nanoparticles. The temporary functional group at N-1 position must be stable throughout the synthetic process, yet must be able to be finally modified and coupled with tethers. The pro-drugs can then be conjugated through the tethers' structure to other macromolecules, such as a dendrimer nanoparticle, to enhance its retention time in vivo that can aid in situations that need prolong drug release. Commercially available 5-methoxy-2-methylindole was first treated with Vilsmeier reagents to give 3-formyl compound **4** with excellent yield. Alkylation of position 1 was then carried out with sodium hydride and ethyl bromo-acetate. Nitration at the desired 4-position could only be achieved at a low reaction concentration and with a large excess of nitric acid. However, a 6-nitro isomer was presented at a low ratio (about 15%). The mixture had poor solubility in organic solvents, making it impossible to purify through chromatography, and when reduced with Sn/HCl gave a mixture of 4- and 6-amine products. The desired 4-amino compound **7** was then purified with chromatography. Fremy's salt was used to covert the aromatic ring to a quinone structure with quantitative yield. The crucial step is the reduction of the 3-carboxaldehyde to a hydroxyl group because there are several other sites can also be reduced in the structure, especially the ethyl ester. The



Scheme 2. Synthesis of indolequinone linker. Reagents and conditions: (i) DMF/$POCl_3$; (ii) NaH/DMF/$BrCH_2CO_2Et$; (iii) AcOH/HNO_3; (iv) Sn/HCl/EtOH, reflux; (v) Fremy's salt/acetone; (vi) $NaBH_4$/MeOH/THF; (vii) LiOH/MeOH/THF; (viii) mono-Bocdiamine or azido amine, HATU, Et_3N, DMF.



Scheme 3. Synthesis of pro-drugs **1**, **2** and **3**. Reagents and conditions: (i) (a) 4-nitrophenyl chloroformate/Et_3N; (b) excess *N*,*N*′-methylethylenediamine; (ii) 4-nitrophenyl chloroformate activated naloxone, Et_3N/CH_2Cl_2; (iii) (a) Triphosgene; (b) Naloxone, triethylamine/dichloromethane.

selective reduction of the aldehyde could only be achieved by using 5 equiv of $NaBH_4$ at 0 °C over 8 min, and any elevation of temperature or prolonging the reaction time decreased the yield drastically. The yield of the reduction reaction was around 60%. After the hydrolysis of the ester with 1 equiv of LiOH, compound **10** was then coupled with a mono-Boc protected diamine to produce the indolequinone linker **11**. An azido amine tether was also coupled to **10** to give indolequinone linker **12** with a tether amenable to 'click' chemistry via an alkyne functional group through 1,3-Huisgen cyclic addition.

The synthesis of all the pro-drugs is shown in Scheme 3. Pro-drug **1** was first prepared from compound **11** which has a carbonate bond between the indolequinone structure and naloxone, however, the carbonate structure is unstable in phosphate buffer saline (PBS) which indicates that it would not be stable in blood. This would lead to no-specific naloxone release. To enhance the stability of the pro-drugs, a small spacer was incorporated between the indolequinone structure and the drug through two carbamate bonds. The carbamate bonds in pro-drugs **2** and **3** are much more stable so undesired release of naloxone can be avoided. In the mean time, the spacer can reduce the steric interference between the drug and the linker and thus enhance enzyme access to the cleavage sit and facilitate hypoxic release of the drug. To achieve this, the activated 4-nitrophenyl carbonate of compounds **11** and **12** were condensed with an excess of *N*,*N*′-methylethylene diamine to give the compounds **13** and **14**. Despite only a 3 equiv of diamine being used in the reactions there was no significant dimerization. The secondary amine at compounds **13** and **14** was then converted to a carbomyl chloride with triphosgene, which was subsequently reacted with naloxone to form the naloxone pro-drugs **2** and **3**. All compounds are characterized with NMR and MS spectra (see Supplementary data).

The hypoxia-induced drug release was characterized and monitored using a Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Briefly, HPLC analysis was carried out on a Waters Delta 600 HPLC system equipped with a Waters 2996 photodiode array (PDA) detector, a Waters 717 Plus auto sampler, and a Waters Fraction collector III. The instrument is controlled by Empower 2 software. For analysis, aliquots were taken from the incubated samples and run on a C5 silica-based RP-HPLC column (250 × 4.6 mm, 300 Å) connected to a C5 guard column (4 × 3 mm). The mobile phase used for elution was a linear gradient beginning with 90:10 (v/v) water/acetonitrile at a flow rate of 1 mL/min. Trifluoroacetic acid at a 0.14 wt % concentration in water as well as in acetonitrile was used as a counter ion. The data were collected at λ_{max} = 280 nm. The HPLC profiles of the pro-drugs and free naloxone are shown in Figure 1. The retention time of free naloxone is at 8.5 min, and the retention times for pro-drug **2** and **3** are 17.4 and 16.6 min, respectively. To quantify the released drug, a calibration curve for free naloxone was generated, and the detection limit of free naloxone using this technique is 8 ng/μL. The pro-drugs were dissolved in DMSO and then diluted with PBS buffer to make the working stock solutions at a concentration of 100 mM for the stability and release studies. Stability of the pro-drugs under non-hypoxic conditions was tested by incubation in PBS buffer. The HPLC results showed that pro-drug **1** was hydrolyzed within 2 h (data not shown). However, pro-drug **2** and **3** were stable in PBS buffer with no free drug detected after 24 h at 37 °C.

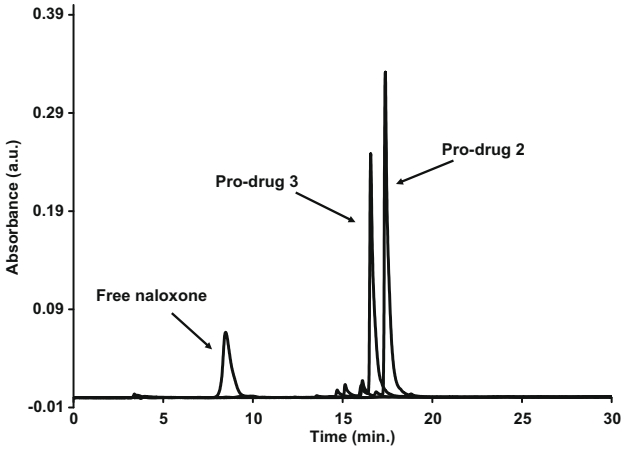


Figure 1. A HPLC chromatogram showing profiles of naloxone and prodrugs. Free naloxone, pro-drug **2** and **3** eluted at 8.5, 17.4 and 16.6 min, respectively.

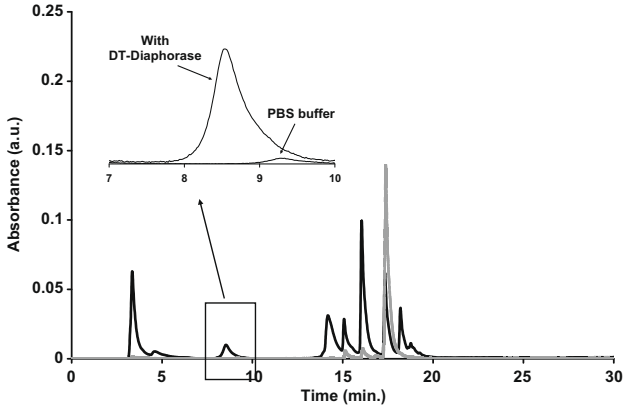


Figure 2. HPLC of naloxone release for pro-drug **2** in the presence of DT-Diaphorase.

The release of naloxone from the pro-drugs was first examined in the presence of the enzyme DT-Diaphorase (DTD; NQO1) in PBS buffer. DTD is an obligate 2-electron reductase that is characterized by its ability to use either NADH or NADPH as a cofactor.[15] A 10 μL 4 mM pro-drug solution was diluted with 180 μL phosphate buffer, a 5 μL 2 mM NADH coenzyme and a 5 μL 40 mU/μL NQO1 solution in PBS buffer (pH 7.0) were added. The final concentration of pro-drugs was 200 μM and the mixture was incubated at 37 °C for 24 h and the result showed that 78% of the naloxone was released for pro-drug **2** and 82% for pro-drug **3** (see Figs. 2 and 3). The identity of the naloxone peak at 8.5 min was confirmed by their UV profiles as well as mass spectra. The structure-bioreductive–activity relationship of a series of indolequinone compounds has been studied by Moody[16] Naylor,[11] and Stratford.[17] They synthesized a series of 2- and 3-substituted indolequinone compounds and evaluated their release activities. The results demonstrated that substitution at the 1-position of the indolequinone nucleus with bulky groups can be tolerated by the enzyme while compounds with an electron-withdrawing substituent at C-3 were better substrates.[13] Our results and observations were in agreement with those previous studies. Pro-drug **2** and **3** has a bulky group at position 1, and the drug moiety is at position 3. Clearly indolequinone binds to the active site in an orientation that allows space for a large substitute in the vicinity of the N-1 position.

We further tested the release of drug from pro-drug **2** in fresh human plasma under conditions of low-pressure oxygen. An air-sealed chamber was made to simulate a hypoxic environment to test the drug release kinetics. An oxygen gauge was installed inside the chamber to determine oxygen pressure levels. Human blood was collected in Citrate Dextrose Solution anticoagulant. The blood was centrifuged at 4 °C (7000*g* for 5 min) to collect the plasma. Pro-drug **2** (final concentration, 125 μM) was mixed with PBS buffer and 30% human plasma. Hypoxic condition was established by degassing the sample with argon slowly for 20 min. After degassing, the partial pressure of oxygen (pO_2) value was measured using a blood gas analyzer. This protocol allowed us to achieve a pO_2 value of 18 mm Hg. After the initial setting of these conditions, the sample vials were degassed and immediately closed and transferred to an air-tight chamber that is previously set under hypoxic conditions. Hypoxia was maintained by passing argon gas through the chamber throughout the sample incubation time. Simultaneously, a control experiment was carried out under normal atmospheric conditions without any degassing procedure. Samples (30 μL each) were withdrawn from each mixture at certain time points, namely 1, 2, 5, and 24 h, and were immediately frozen in dry ice. Samples were thawed prior to HPLC analysis. The time course of the naloxone release under hypoxic conditions is shown in Figure 4. It is noteworthy that 64% of the drug was released in the first 5 h, and after 24 h, almost all the naloxone (99.2%) was released form pro-drug **2**. However, the drug release was completely inhibited under normoxic conditions (Fig. 4). This remarkable difference suggests that the drug release rates are dependent on the oxygen level in the reaction media, which further indicates that the release kinetics involve an intermediate $Q^{\cdot-}$ radical. The radical is very reactive toward oxygen, thus the half-life time of the species is extremely short and the intermediate will be oxidized back to quinone under a higher oxygen level, which will not release the drug.[18] The results suggest that under hypoxic conditions, reductive enzymes in blood plasma can catalyze the release the naloxone from the pro-drug through a bio-reductive mechanism.

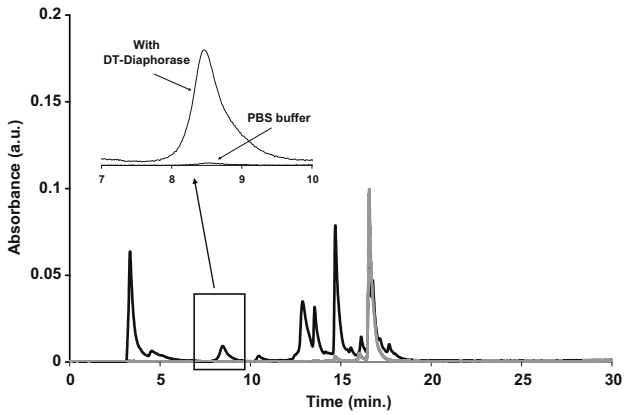


Figure 3. HPLC of naloxone release for pro-drug **3** in the presence of DT-Diaphorase.

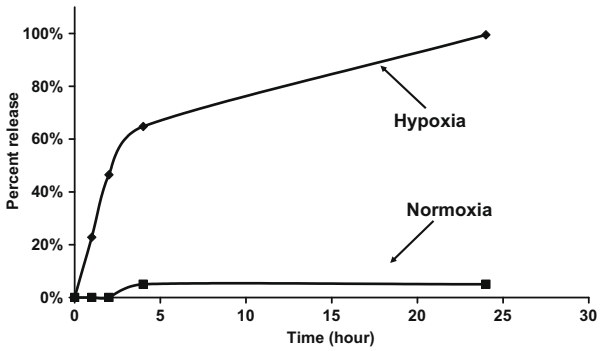


Figure 4. HPLC time course of naloxone release for pro-drug **2** under hypoxic and normoxia conditions in 30% human plasma.

In conclusion, we have successfully synthesized naloxone pro-drugs bearing an indolequinone structure that enzymatically releases free naloxone. The results of this study suggest that pro-drug **2** may be useful in a narcotic antagonist feedback system releasing free naloxone, when hypoxic conditions occur, such as in respiratory failure. Furthermore, a long chain spacer at indole position 1 may serve as a reactive point for conjugation of these pro-drugs to macromolecules to further modify the pharmacokinetics of these pro-drugs in vivo.

Acknowledgement

Financial support from Defense Advanced Research Projects Agency (DARPA 53270-MS-DRP) is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.061.

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