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Synthesis and Cytotoxicity of 5-Fluorouracil/Diazeniumdiolate Conjugates

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Abstract—5-Fluorouracil/diazeniumdiolate conjugates were first synthesized, and showed greater cytotoxicities than 5-fluorouracil for DU 145 human prostate and HeLa cancer cells.

Introduction

Nitric oxide (NO) plays a critical role in a variety of bioregulatory processes, including normal physiological control of the blood pressure, neurotransmission, and microphage-induced cytostatics and cytotoxity.[1] Recently, conjugation of NO releasing moieties with active drugs is of particular interest. Studies showed that the use of non-steroid anti-inflammatory drug derivatives (NSAID) with NO releasing properties had improved gastrointestinal safety.[2] There are several NO-donating NSAIDs, for example, NO-Aspirin and NO-Flurbiprofen, in clinical trials at present. Moreover, NO can inhibit metastasis, enhance cancer cell apoptosis and assist macrophage to kill tumor cells.[3]

Diazeniumdiolates (NONOates) are compounds containing the $[N(O)NO]^-$ structural unit. This class of compounds is known as an excellent source for a controlled release of NO both in vitro and in vivo.[4] Keefer's group has recently introduced a new class of diazeniumdiolate prodrugs which are acetoxymethylated at the anionic oxygen of the parent NONOates. These esterase-sensitive compounds reveal significant antileukemic activity in vitro.[5]

5-Fluorouracil (5-FU, Fig. 1) is one of the antitumor agents most frequently used for treating solid tumors, such as breast, colorrectal, and gastric cancers. To optimize the efficacy of 5-FU, it is often administrated by continuous infusion as well as incombination with other cytotoxic or with biochemical modulators, such as leucovorin.[6] 5-FU is poorly tumor selective, so its therapy causes high incidences of toxicity in the bone marrow, gastrointestinal tract, central nerve system and skin, which has prompted the efforts in the development of derivatives aiming at reducing the adverse effects of 5-FU. Novel prodrugs of 5-FU possessing a broader spectrum of antitumor activity and fewer toxicity have been sought diligently by many researchers.[7,8] The common feature of these prodrugs is that they are all N^1-modified derivatives through different biodegradable linkages.[9]

Based on the mutual prodrug concept, 5-FU and diazeniumdiolate conjugates (Fig. 1) with methylene or acyloxymethylene as the spacers were synthesized with the aim to improve tumor selectivity, efficiency and safety. Their NO-releasing ablilties and cytotoxicity were also evaluated.

Results and Discussion

Synthesis of 5-FU/diazeniumdiolate conjugates

The synthetic scheme toward the conjugate **1** is shown in Scheme 1. The sodium PYRRO/NO **3**, was synthesized according to a modified procedure reported by Keefer.[10] The chloromethyl NONOate **5** was obtained

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from the *O*-alkylation of sodium PYRRO/NO **3** with chloromethyl methyl sulfide and subsequent treatment with sulfuryl chloride.[11] Attachment of 5-FU to **5** gave the desired N^1-alkylation product **1** as the major product. The N^1,N^3-bis-alkylation product **1a** was also obtained from the same reaction.

The stepwise synthesis of conjugate **2** is illustrated in Scheme 2. The intermediate **10** could be obtained following a similar procedure reported by Menger and Rourk.[12] Alkylation of succinic acid monobenzyl ester (**6**) with chloromethyl methyl sulfide and subsequent treatment with sulfuryl chloride generated the desired chloromethyl ester **8**. Attachment of 5-FU to **8** provided the compound **9** as the only product, and there was no sign of the N^3-alkylation product. Catalytic transfer hydrogenation debenzylation of **9** in glacial acetic acid, using 1,4-cyclohexadiene as the hydrogen source, gave **10** in quantitative yield. Coupling of **10** with chloromethyl PYRRO/NO **5** gave the desired N^1-alkylation product **2**, along with the N^1,N^3-bis-alkylation product **2a**.

Another previously reported prodrug AcOM-PYRRO (**11**) was synthesized from **5** in high yield by a modified procedure (Scheme 3).[5]

Hydrolysis of conjugates

The hydrolysis of the prodrugs were studied by monitoring of the NO release with an Electrochemical ISO-NO marker II isolated Nitric Oxide Meter manufactured by World Precision Instruments, Inc. (Sarasota, Florida). All of the prodrugs **1**, **2**, and **11** are stable in aqueous solution at neutral pH. Both **2** and **11** slowly release NO at pH = 8 (Fig. 2), and in the presence of porcine liver esterase (Fig. 3). The NO release rate for compound **2** (Scheme 4) is much slower than **11** at the same condition. Compound **2** should be subjected to esterase action for longer than 3 min. Scheme 4 shows the proposed NO releasing pathway. On the contrary, the produrg **1** was very stable in aqueous solution. No substantial NO was detected even at either acidic or basic conditions. There might be other enzymatic pathways to release 5-Fu and NO. Further experiments are necessary to clarify the mechanism.

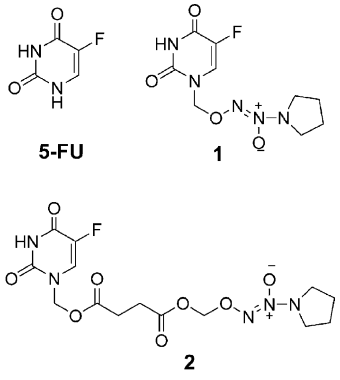
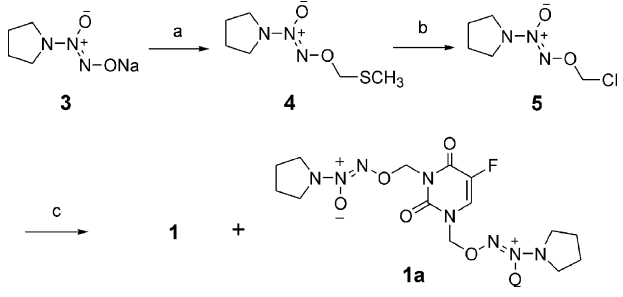


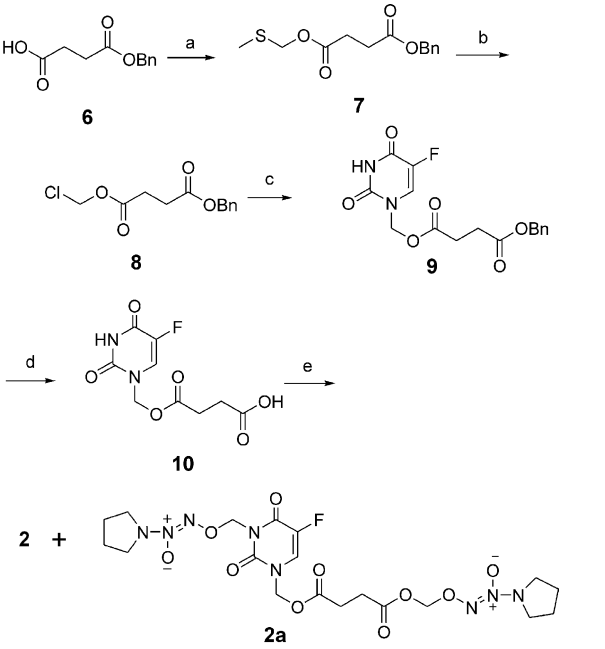
Figure 1.



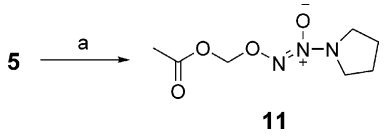
Scheme 1. (a) $ClCH_2SCH_3$, KI, DMF/THF, rt (45%); (b) SO_2Cl_2, CH_2Cl_2, rt ; (c) 5-FU, NEt_3, DMF, rt (**1**, 21%; **1a**, 12%).

Cytotoxic effects

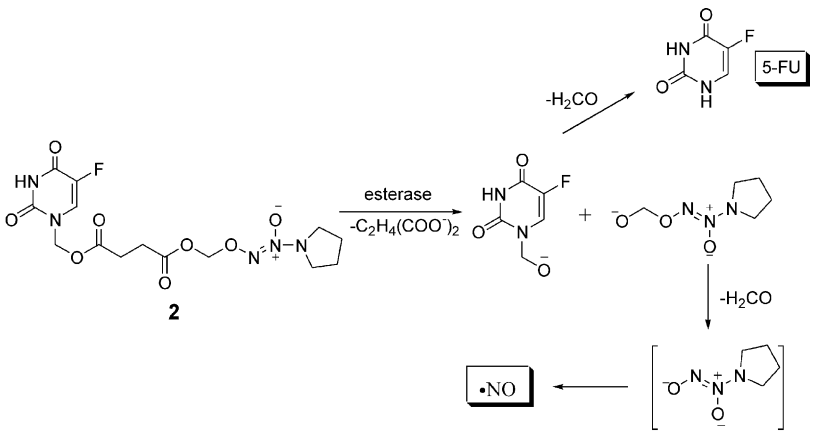
The cytotoxicities of 5-FU/diazeniumdiolate conjugates were assessed by in vitro clonogenic cell survival assays as described previously (Table 1).[13] The median effect dose (ED50) for compound **1** in DU145 (98 μM) and HeLa (112 μM) cell lines indicated that this prodrug was about twice as potent as 5-FU (204 μM in DU145 and 278 μM in HeLa). Compound **2a** (257 μM) and 5-FU (278 μM) exhibited similar cytotoxic effects in HeLa cells, however compound **2** (50 μM) was twice as potent as compound **1** and 5-fold more toxic than 5-FU and compound **2a**. In DU145 cells, compound **2a** (65 μM) exhibited the greatest cytotoxicity. It was about twice as potent as compound **2**, and about three fold more



Scheme 2. (a) $ClCH_2SCH_3$, Cs_2CO_3, DMF, rt (88%); (b) SO_2Cl_2, CH_2Cl_2, rt (70%); (c) 5-FU, NEt_3, DMF, rt (54%); (d), 10%Pd/C, AcOH, 1,4-cyclohexadiene, rt (100%); (e) **5**, Cs_2CO_3, DMF, rt (**2**, 18%; **2a**, 12%).



Scheme 3. (a) NaAc, DMF, rt (51%).



Scheme 4. The hydrolysis pathway of conjugate **2**.

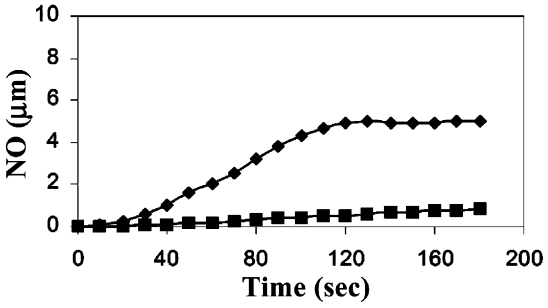


Figure 2. NO releasing from prodrugs **2** (■) and **11** (◆) in a pH = 8.0 buffer.

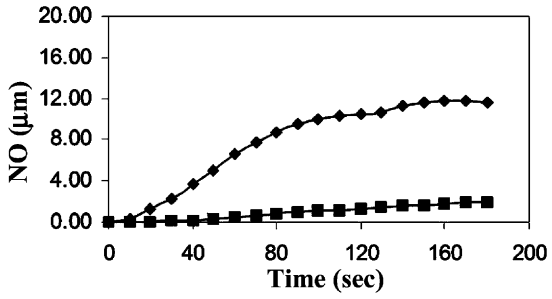


Figure 3. NO releasing from prodrugs **2** (■) and **11** (◆) in the presence of esterase.

Table 1. Median effect dose (μM) for HeLa and DU145 cell lines

Compd	HeLa	DU145
5-FU	278	204
1	112	98
2	50	129
2a	257	65

potent than 5-FU. Compound **11** had little or no effect in the tested cell lines.

Since both of the conjugate **1** and **2** have greater activity than 5-FU, the anti-tumor nucleoside and NO could have synergetic effects in biological systems. The apparent difference between the cytotoxic activities of the two conjugates, **1** and **2**, could be explained by the appreciation of the individual design. The N–O ketal linker in compound **1** could not be hydrolyzed as easy as the spacer in compound **2**.

Experimental

Chemistry

All reagents were purchased from commercial suppliers and were used without further purification. Melting points were measured on an Electrothermal IA 9100 digital apparatus without correction. ^{1}H and ^{13}C NMR were recorded on a Varian Unity-300, a Mercury-400, or a Varian unity-500 spectrometer. MS spectra were obtained from a Kratos MS 80 spectrometer using electronspray ionization mode (ESI). UV–vis spectroscopy measurements were carried out on a HP 8453 spectrometer. Silica gel F_{254} plates (Merck) and Silica Gel 60 (70–230 mesh) Merck) were used in analytical thin-layer chromatography (TLC) and silica gel column chromatography, respectively.

Sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (3). A modified procedure was adopted from ref 5. A solution of 16.5 mL (0.20 mol) of pyrrolidine in a mixture of diethyl ether (40 mL) and acetonitrile (12 mL) was placed in a reaction flask, then bubbled with nitrogen for 20 min, and cooled to −78 °C using a dry ice/acetone bath. Then NO gas was slowly bubbled into the flask for 1 h. The bath was removed, and the reaction vessel was flushed with nitrogen to remove the NO gas. After 10 min, 100 mL of dry diethyl ether was added into the mixture, and a white precipitate was obtained after the filtration under nitrogen. To the white solid was added 10 mL of aqueous NaOH solution (10 M). The mixture was stirred and 100 mL of diethyl ether was added. The white solid was collected by filtration and washed with ether. The product was dried under vacuum to give 5.6 g (37%) yield of **3**. ^{1}H NMR was the same as the literature.[5]

***O*^{2}-Methylthiomethyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (4).** A slurry of compound **3** (3.20 g, 20.9 mmol) and anhydrous sodium carbonate (2.22 g, 20.9

mmol) in 40 mL of dry THF was cooled with an ice-water bath, to which 2.28 mL (27.2 mmol) chloromethyl methylsulfide was slowly added via a syringe, followed by 15 mL of DMF. After 5 min, the bath was removed, and catalytic amount of KI (200 mg) was added. The reaction mixture was stirred under nitrogen overnight. Then it was diluted with 200 mL of ether, washed with cold water. The separated aqueous layer was extracted with ether again. The combined ether solution was washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum. The residue was purified by silica gel chromatograph with 1:4 ethyl acetate/hexane, resulting in 1.80 g (45%) of **4** as colorless oil, which solidified when kept inside refrigerator. R_f 0.38 (EtOAc/hexane: 1/3). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.09 (s, 2H), 3.44 (t, 4H), 2.15 (s, 3H), 1.83 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 78.06, 50.94, 23.02, 15.61. MS 214 (M + Na), 405 (2M + Na). HRMS calcd for $C_6H_{13}N_3O_2S$ 191.0728, found 191.0727.

O^2-Chloromethyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (5). A solution of compound **4** (91 mg, 0.48 mmol) in 10 mL of dry CH_2Cl_2 was cooled with an ice-water bath, to which 0.48 mL of sulfuric chloride (1.0 M in CH_2Cl_2) was added dropwise. After 10 min, the ice-water bath was removed, and the mixture was stirred for another 30 min. Evaporation under vacuum gave **5** as yellow oil, which was immediately used in the following reactions without further purification. R_f 0.43 (EtOAc/hexane: 1/3). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.80 (s, 2H), 3.60 (t, 4H), 1.95 (m, 4H); ^{13}C NMR (CDC_{l3}, 100 MHz): δ 79.86, 50.86, 23.32.

5-FU/diazeniumdiolate conjugate (1). To a solution of 5-fluorouracil (133 mg, 1.02 mmol) in 5 mL of DMF was added triethyl amine (328 μL, 2.36 mmol), the mixture was stirred at room temperature for 30 min. Then a solution of compound **5** in 5 mL DMF, which was freshly prepared from **4** (150 mg, 0.78 mmol) and sulfuric chloride (0.78 mL, 1.0 M in CH_2Cl_2), was added dropwise, and the mixture was stirred overnight. The mixture was diluted with 100 mL of water, extracted with CH_2Cl_2 twice (50 mL×2). The separated organic phase was combined, washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum. The residue was loaded on the silica gel chromatograph column. Elution with 1:1 ethyl acetate/methylene chloride provided 45 mg (21%) **1** and 50 mg (12%) **1a**. **1**: Colorless solid, mp 172–178 °C; R_f 0.26 (EtOAc/CH_2Cl_2: 1/1); ^{1}H NMR (acetone-d_6, 400 MHz): δ 7.98 (d, 1H, J = 6.4 Hz), 5.67 (s, 2H), 3.53 (m, 4H), 1.95 (m, 4H); ^{13}C NMR (acetone-d_6, 100 MHz): δ 129.53, 129.20, 78.13, 51.26, 23.39. MS (ESI) 296 ($M + Na^+$), 569 ($2M + Na^+$); HRMS calcd for $C_9H_{12}FN_5O_4$ 274.0952, found 274.0962 (M + H). **1a**: R_f 0.45 (EtOAc/CH_2Cl_2: 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.43 (d, 1H, J = 5.0 Hz), 5.99 (s, 2H), 5.67 (s, 2H), 3.55 (m, 4H), 1.95 (m, 4H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 126.36, 126.09, 77.78, 71.17, 71.17, 50.65, 50.56, 23.04, 22.89. MS (ESI) 439 ($M + Na^+$), 855 ($2M + Na^+$).

Succinic acid benzyl ester (methylsulfanyl)methyl ester (7). To the stirred solution of succinic acid monobenzyl ester (4.0 g, 19.0 mmol) in 25 mL of DMF was added cesium carbonate (6.2 g, 19.0 mmol), the resulting suspension was stirred for 30 min at room temperature. Then chloromethyl sulfide (1.59 mL, 19.0 mmol) was added, and the mixture was stirred at room temperature overnight and at 70 °C for 10 min. The mixture was cooled to room temperature, diluted with water, and extracted with ethyl acetate. The separated organic layer was washed with brine, dried over sodium sulfate, and concentrated to give 4.5 g (88%) yellow oil. R_f 0.35 (EtOAc/hexane: 1/3); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.34 (m, 5H), 5.12 (4H), 2.69 (4H), 2.21 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.88, 135.62, 128.50, 128.24, 128.18, 68.43, 66.54, 29.12, 28.96, 15.31.

Succinic acid benzyl ester chloromethyl ester (8). Following similar procedure for the synthesis of **5**. 70% yield after flash column chromatography. R_f 0.38 (EtOAc/hexane: 1/3); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.35 (m, 5H), 5.68 (s, 2H), 5.14 (s, 2H), 2.72 (4H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.88, 170.70, 135.83, 128.83, 128.59, 128.52, 69.00, 66.96, 66.91, 64.22, 29.19, 28.98. MS (ESI) 279 ($M + Na^+$).

Succinic acid benzyl ester (5-fluro-2,4-dioxo-di-hydro-2H-pyrimidin-1-yl)methyl ester (9). Following similar procedure for the synthesis of **1**. 54% yield after flash column chromatography; white solid, mp 125–127 °C; R_f: 0.72 (EtOAc/CH_2Cl_2: 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.04 (br, 1H), 7.56 (d, J = 4.5 Hz, 1H), 7.35 (m, 5H), 5.62 (s, 2H0, 5.12 (s, 2H), 2.70 (s, 4H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 172.54, 171.73, 156.83, 149.09, 141.09, 139.18, 135.50, 128.59, 128.39, 128.29, 128.23, 69.75, 66.78, 28.80. MS (ESI) 373 ($M + Na^+$), 723 ($2M + Na^+$).

Succinic acid mono[(5-fluro-2,4-dioxo-di-hydro-2H-pyrimidin-1-yl)methyl] ester (10). To the stirred solution of compound **9** (470 mg, 1.34 mmol) in 10 mL of acetic acid was added 10% Pd/C (0.5 g) and 1,4-cyclohexadiene (0.76 mL, 8.0 mmol). The mixture was stirred for 3 h at room temperature, then it was filtered and evaporated to give 350 mg product **10** (100%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.08 (d, J = 6.4 Hz, 1H), 5.55 (s, 2H), 2.48 (m, 4H). MS (ESI) 283 ($M + Na^+$), 543 ($2M + Na^+$).

5-FU/diazeniumdiolate conjugate (2) and (2a). To a solution of 5-fluorouracil (145 mg, 0.56 mmol) in 5 mL of DMF was added cesium carbonate (182 mg, 0.56 mmol). The mixture was stirred at room temperature for 30 min. Then a solution of compound **5** in 5 mL DMF, which was freshly prepared from **4** (128 mg, 0.67 mmol) and sulfuric chloride (0.67 mL, 1.0 M in CH_2Cl_2), was added dropwise, and the mixture was stirred overnight. After the evaporation of solvent under vacuum, the residue was loaded onto a silica gel chromatograph column. Elution with 1:1 ethyl acetate/methylene chloride provided 40 mg (18%) **2** and 36 mg (12%) **2a**. **2**: R_f 0.45 (EtOAc/CH_2Cl_2: 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.38 (br, 1H), 7.60 (d, J = 6.4 Hz, 1H), 5.72 (s, 2H), 5.62 (2H), 3.59 (m, 4H), 2.66–2.73 (m, 4H), 1.96 (m, 4H); ^{13}C NMR ($CDCl_3$, 125 MHz):

δ 171.2, 170.93, 149.17, 141.03, 139.14, 128.69, 128.42, 87.44, 87.30, 69.93, 50.69, 50.56, 28.78, 28.55, 23.02, 22.94. MS (ESI) 426 ($M+Na^+$), 829 ($2M+Na^+$); HRMS (FAB) calcd for $C_{14}H_{19}FN_5O_8$ (M + H) 404.1218, found 404.1225 (M + H). **2a**: R_f: 0.58 (EtOAc/CH_2Cl_2: 1/1); ^1H NMR ($CDCl_3$, 500 MHz): δ 7.63 (d, 1H, $J = 5.0$ Hz), 5.96 (s, 2H), 5.74 (s, 2H), 5.66 (s, 2H), 3.56 (m, 4H), 3.52 (m, 4H), 1.95 (m, 4H), 1.91 (m, 4H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 172.19, 170.55, 128.17, 127.77, 127.51, 87.40, 71.17, 70.78, 50.63, 28.74, 28.65, 28.52, 23.00, 22.92, 22.85. MS (ESI) 569 ($M+Na^+$), 1115 ($2M+Na^+$). HRMS (FAB) calcd for $C_{19}H_{27}FN_8O_{10}Na$ (M + Na) 569.1732, found 569.1752.

***O*²-(Acetoxymethyl) 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (11).** To a DMF (5 mL) solution of freshly prepared compound **5**, which was obtained from **4** (110 mg, 0.58 mmol) and sulfuric chloride (0.58 mL, 1.0 M in CH_2Cl_2), was added sodium acetate (158 mg, 1.16 mmol) in one portion. The mixture was stirred at room temperature for 7 h, then it was diluted with 100 mL of water, extracted with CH_2Cl_2 twice (50 mL×2). The separated organic phase was combined, washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum to give 60 mg (51%) solid. ^1H NMR was the same as the literature.[5] ^{13}C NMR ($CDCl_3$, 100 MHz): δ 129.99, 87.36, 50.71, 22.98, 20.91.

Nitric oxide measurement

NO measurement was carried out with an Electrochemical ISO-NO Mark II Isolated Nitric Oxide Meter, a product of World Precision Instruments, Inc. (Sarasota, FL, USA). For the test in pH 8.0 buffer, the prodrug was dissolved in methanol and added to 10 mL of 1.0 M Tris–Cl buffer (pH = 8.0) to a final concentration of 0.10 mM. For the enzymatic test, the prodrug was dissolved in methanol and added to 10 mL of 0.1 M Tris–Cl buffer (pH = 7.0) to a final concentration of 0.10 mM. Then, esterase (EC.31.1.1, procine liver, crude, Sigma) was added to a final concentration of 150 μg/mL.

Clonogenic cell survival assay

Cell lines. DU-145, human prostate adenocarcinoma and HeLa, human cervical adenocarcinoma were maintained in RPMI 1640, supplemented with 15% heat inactivated fetal bovine serum and 1% penicillin-streptomycin liquid (Life Technologies). 2×10^5 cells were seeded into T-25 tissue culture flasks 24 h before treatment with the compounds.

Media was removed from the cells then washed one time with Hanks balanced salt solution (HBSS). Three millilitres of HBSS were placed on cells for treatment period. Treatment time was 2 h at 37 °C and 5% CO_2. Stock solutions were prepared at a 0.01 M concentration and sterilized by passing through a 0.2 micron filter. Solvents used to prepare the solutions are as follows: 5-FU in distilled sterile water; TXP30 in DMSO; other compounds in ethanol.

Cells were washed one time with HBSS, removed from flasks with trypsin, counted and plated at 50, 100, 250 cells into six-well tissue culture plates, containing cell culture media. Cells were incubated for seven to ten days at 37 °C and 5% CO_2, fixed with methanol, stained with Giemsa, and colonies greater than 50 cells counted. Several concentrations of the compounds were used to assess the dose dependent cytoxicity by clonogenic cell survival, calculated as described previously.[13] Experiments were run two times and averages calculated. Graphs and statistics were calculated using Sigma Plot software.

Acknowledgements

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References and Notes

1. Ignarro, L. J. *Nitric Oxide: Biology and Pathobiology*; Academic: San Diego, 2000.
2. Burgaud, J. L.; Riffaud, J. P.; Del Soldato, P. *Curr. Pharm. Des.* **2002**, *8*, 201.
3. Jia, Q.; Janczuk, A. J.; Cai, T.; Xian, M.; Wen, Z.; Wang, P. G. *Expert Opin. Ther. Pat* **2002**, *12*, 819.
4. Hrabie, J. A.; Keefer, L. K. *Chem. Rev.* **2002**, *102*, 1135.
5. Saavedra, J. E.; Shami, P. J.; Wang, L. Y.; Davies, K. M.; Booth, M. N.; Citro, M. L.; Keefer, L. K. *J. Med. Chem.* **2000**, *43*, 261.
6. Machover, D. *Cancer* **1997**, *80*, 1179.
7. Malet-Martino, M.; Martino, R. *Oncologist* **2002**, *7*, 288.
8. Malet-Martino, M.; Jolimaitre, P.; Martino, R; *Curr. Med. Chem.: Anti-Cancer Agents* **2002**, *2*, 267.
9. Shimma, N.; Umeda, I.; Arasaki, M.; Murasaki, C.; Masubuchi, K.; Kohchi, Y.; Miwa, M.; Ura, M.; Sawada, N.; Tahara, H.; Kuruma, I.; Horii, I.; Ishitsuka, H. *Bioorg. Med. Chem.* **2000**, *8*, 1697.
10. Saavedra, J. E.; Billiar, T. R.; Williams, D. L.; Kim, Y.-M.; Watkins, S. C.; Keefer, L. K. *J. Med. Chem.* **1997**, *40*, 1947.
11. Hou, Y.; Xie, W.; Janczuk, A. J.; Wang, P. G. *J. Org. Chem.* **2000**, *65*, 4333.
12. Menger, F. M.; Rourk, M. J. *J. Org. Chem.* **1997**, *62*, 9083.
13. Braunschweiger, P. G.; Basrur, V. S.; Cameron, D.; Sharpe, L.; Santos, O.; Perras, J. P.; Sevin, B. U.; Markoe, A. M. *Biotherapy* **1997**, *10*, 129.