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Synthesis and biological evaluation of nitric oxide-releasing hybrids from gemcitabine and phenylsulfonyl furoxans as anti-tumor agents

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Abstract

A series of novel hybrids **10a-m** were designed and synthesized by coupling phenylsulfonyl furoxans with gemcitabine through various diols or alcohol amines linkers, and their biological activities were evaluated *in vitro*. Most of hybrids exhibited good to moderate anti-tumor activities, which are associated with NO release. In particular, hybrid **10e** showed excellent anticancer activities which were more potent than or comparable to gemcitabine. However, inhibition of nucleoside transport only significantly decreased the inhibitory rates of gemcitabine against HepG2 cells, but not **10e**, and the inhibitory rates of **10e** were partially reduced by pre-treatment with hemoglobin, demonstrating that the anti-tumor activity of **10e** might result from the synergic effect of high levels of NO production and gemcitabine fragment. In addition, compound **10e** could apparently induce cell apoptosis by regulating apoptotic relative proteins. Therefore, our novel findings provide a proof of principle in the design of new furoxan/gemcitabine hybrids for the intervention of human cancers.

Keywords: Nitric oxide; Hybrids; Phenylsulfonyl furoxans; Gemcitabine; Anti-tumor agents

Introduction

Cancer is one of the most serious diseases threatening human health in the world. Current anti-tumor agents are still yet to meet human use requirements of high efficiency and low toxicity. Various nucleoside analogue drugs are widely used for the treatment of cancer.[1] Gemcitabine (2′, 2′-difluorodeoxycytidine, dFdC), a cycle-specific nucleoside analogue, has been approved for the treatment of various solid tumors,[2] such as non-small-cell lung cancer (NSCLC), pancreatic cancer, ovary cancer, and breast cancer.[3-7] However, there are some drawbacks of gemcitabine in clinical use, including poor fat-solubility, poor transmembrane penetration, degraded deaminase inactivation, short half-life, low bioavailability, and drug resistance.[8] Hence, it is urgent for researchers to design novel gemcitabine derivatives to further improve its properties.

Based on the literature, several approaches have been applied to the structural modification of gemcitabine to improve its properties.[9,10] It is more common that the N^4-position of the cytidine ring of gemcitabine be modified to form a hydrolysable amide linkage. For example, the orally available N^4-valproyl gemcitabine (LY2334737, **Figure 1**), which is currently in phase I clinical

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trial, could avoid the fast degradation of gemcitabine and withstand the gastrointestinal environment with different pH values.[11,12] N^4-Squalenoyl gemcitabine (SQdFdC) was stable in plasma and its half-life of metabolism has been extended in vivo.[13] In addition, stearoyl gemcitabine (C18dFdC), the N^4-stearoyl substituted gemcitabine, could prevent the degradation of gemcitabine by deaminase and therefore improved the bioavailability and half-life of gemcitabine.[14]

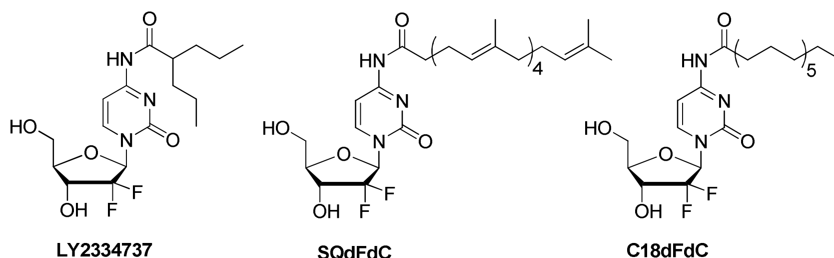

Fig. 1. Chemical structures of gemcitabine derivatives.

Nitric oxide (NO) as a signaling and/or effector molecule plays a pivotal role in numerous physiologic and pathologic processes.[15] It is well known that high levels of NO generated from NO-donors can not only induce apoptosis and inhibit metastasis of tumor cells, but also sensitize tumor cells to chemotherapy, radiation, and immunotherapy *in vitro* and *in vivo*.[16,17] Furoxans, especially phenylsulfonyl furoxans, represent an important class of lipophilic NO donors and have played an increasingly important role in the development of anti-cancer reagents which can produce high levels of NO and inhibit the growth of tumors *in vivo*.[18-22] Based on the above findings, we hypothesized that hybridization of furoxans with gemcitabine may release high levels of NO to exert synergistic anticancer effects with gemcitabine. According to this hypothesis, a total of 13 novel hybrids **10a-m** were designed and synthesized by coupling phenylsulfonyl furoxans with the N^4-position of gemcitabine through various diols or alcohol amines linkers. Their anti-tumor effects, membrane transport deficiency, NO-releasing ability and effects on key regulatory proteins were evaluated *in vitro*. Herein, we report the synthesis and biological evaluation of target compounds.

Results and discussion

Chemistry

The synthetic route of **10a-m** is presented in **Scheme 1**. The furoxans were synthesized in a four-step sequence. The starting material benzenethiol **1** was converted to 2-(phenylthio) acetic acid **2** by treatment with NaOH and chloroacetic acid and Na_2CO_3 at 80 °C in 89.0 % yield. Compound **2** was treated with 30 % H_2O_2 at room temperature to generate 2-(phenylsulfonyl) aceticacid **3**, which was poured into fuming HNO_3 at 90 °C to produce diphenylsulfonyl furoxan **4** in 76.0% yield. Subsequently, compound **4** was converted to various monophenylsulfonylfuroxans **5a-m** by linking with different diols or alcohol amines. Then **6a-m** were formed by condensation of succinic anhydride with **5a-m**. Gemcitabine **7** was treated with tert-butyldimethylsilyl chloride (TBDMSCl) and imidazole to give intermediate **8**, which was directly reacted with **6a-m**

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in CH_2Cl_2 in the presence of *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride/4-dimethylaminopyridine (NHS/EDCI/DMAP) to generate products **9a-m** in yields of 35-60 %. Finally, compound **9a-m** were further deprotected by tetrabutylammonium fluoride (TBAF) to give target compounds **10a-m**. The final products were purified by column chromatography and their structures were characterized by ^{1}H NMR, ^{13}C NMR, MS, and elemental analyses.

Scheme 1. Reaction conditions and reagents: a) 30 % NaOH, chloroacetic acid, Na_2CO_3, reflux, 1h, 89.0 %; b, c) HOAc, 30% H_2O_2, rt, 2.5h, 95 % HNO_3, 90 °C, 0.5h, 76.0 %; d) diols or alcohol amines, 25 % NaOH or NaH, 0 °C, 0.5 h, 50.0-70.0 %; e) Succinic anhydride, CH_2Cl_2, reflux, 3-8 h, 85.0-92.7 %; f) TBDMSCl, imidazole, DMF, rt, 6 h, 87.3 %; g) NHS, EDCI, DMAP, rt, 18 h, 47.8-59.0 %; h) TBAF, THF, 0 °C, 0.5 h, 66.9-87.2 %.

Biological evaluation

All the target compounds **10a-m** were evaluated in MTT assay against human hepatocellular carcinoma cells (HepG2), human colon cancer cell (HCT-116, SW-620), human lung cancer cell (A549), and human gastric carcinoma cells (SGC7901) in vitro. Gemcitabine was used as a positive control. The IC_{50} values of **10a-m** against each tumor cell line are presented in **Table 1**. All tested compounds exhibited more potent anti-tumor activity, especially compounds **10e-k** (IC_{50} = 1.02~9.92 μM) which possessed similar or better inhibition activities for the cancer cell growth relative to gemcitabine (IC_{50} = 2.69~7.33 μ M). Notably, the IC_{50} of **10e** (1.02 μM) against HepG2 cells was eight-fold lower than its NO moiety **6e** (IC_{50} = 8.73 μM), nearly three-fold lower than gemcitabine **7** (IC_{50} = 2.97 μM), and even lower than the combination of **7** and **6e** (IC_{50} =

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1.85 μM, **Figure 2**). These results suggest that the anti-tumor activity of **10e** may result from the synergetic effects of the NO donor moiety **6e** and the gemcitabine moiety by simultaneously acting on both pathways.

Table 1. The IC_{50} values of **10a-m** against five human cancer cell lines.

Compound	*In vitro* inhibition of human cancer cells proliferation (IC_{50}[a], μM)				
	HepG2	HCT-116	SW-620	A549	SGC7901
Gemcitabine (**7**)	2.97 ± 0.32	7.33 ± 0.63	5.62 ± 0.47	2.69 ±0.28	3.58 ± 0.33
JS-K	7.42 ± 0.59	3.75 ± 0.44	5.16 ± 0.63	ND[b]	ND
6e	8.73 ± 0.71	9.86 ± 0.92	9.09 ± 0.75	>12.5	11.5 ± 1.02
10a	>12.5	10.3 ± 0.80	>12.5	>12.5	>12.5
10b	5.32 ± 0.66	6.89 ± 0.59	5.00 ± 0.61	9.92 ± 1.05	9.26 ± 0.76
10c	2.44 ± 0.27	3.57 ± 0.41	2.49 ± 0.30	5.18 ± 0.46	6.13 ± 0.71
10d	3.25 ± 0.43	4.69 ± 0.32	3.98 ± 0.35	7.82 ± 0.75	7.44 ± 0.68
10e	1.02 ± 0.13	1.19 ± 0.20	1.30 ± 0.16	3.27 ± 0.39	1.36 ± 0.22
10f	>12.5	11.3 ± 1.10	9.67 ± 1.09	>12.5	10.5 ± 1.00
10g	7.05 ± 0.64	7.86 ± 0.80	>12.5	11.5 ± 1.12	10.7 ± 1.21
10h	9.21 ± 0.79	8.78 ± 0.72	>12.5	>12.5	>12.5
10i	10.5 ± 0.87	>12.5	9.16 ± 0.95	>12.5	9.92 ± 1.02
10j	6.18 ± 0.59	4.10 ± 0.46	4.97 ± 0.63	8.90 ± 0.96	6.71 ± 0.58
10k	3.22 ± 0.26	3.06 ± 0.38	4.35 ± 0.51	6.04 ± 0.72	5.10 ± 0.40
10l	8.39 ± 0.70	10.8 ± 1.07	5.21 ± 0.44	10.1 ± 1.05	5.85 ± 0.63
10m	10.7 ± 1.01	>12.5	>12.5	>12.5	11.8 ± 1.12

[a] The inhibitory effects of individual compounds on the proliferation of cancer cell lines were determined by the MTT assay. The data are the mean values of IC_{50} from at least three independent experiments. [b] Not detected.

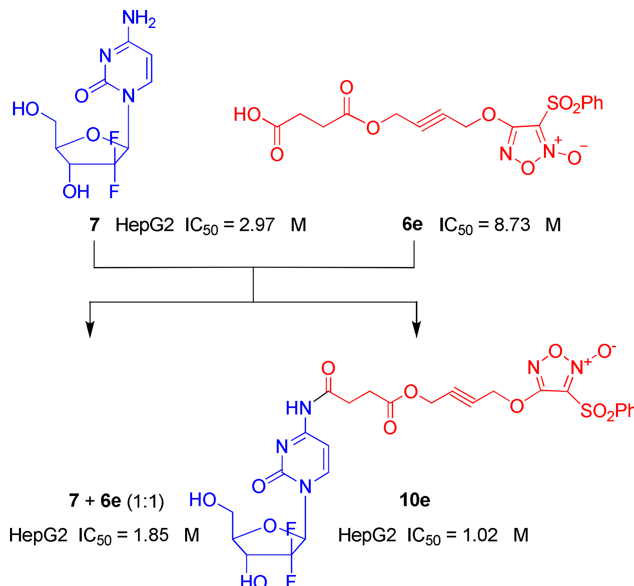

Figure 2. Comparison of anticancer activity of hybrid **10e** with that of **7**, **6e**, and an equimolar mixture of **7** and **6e**.

Given the strong growth inhibitory activity of **10e** *in vitro*, the selectivity profile of **10e** was investigated on the inhibitory effects of HepG2 and LO2 cells using gemcitabine as a control. The treatment with increased dose of **10e** had no significant effect on the survival of non-tumor LO2 cells while the same treatment induced the majority of HepG2 cell death (**Figure 3**), suggesting **10e** may possess selective antiproliferation activity against tumor cells.

Nucleoside transporters have been thought to have an important role in membrane transport systems of gemcitabine. To study the role of membrane transport, the sensitivity to gemcitabine and **10e** in the presence of nucleoside transport inhibitor dipyridamole (Dipy) was determined. As

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shown in **Figure 4**, cell inhibitory rates were determined for gemcitabine and **10e** with different concentrations in HepG2 cells with or without 4 μg/mL dipyridamole. It was found that dipyridamole sharply decreased sensitivity to gemcitabine in HepG2 cells, whereas the sensitivity to **10e** remained almost unchanged. These results suggested that the furoxan/gemcitabine hybrid **10e** may not be dependent on the nucleoside transporter and may independently transport over the cell membrane.

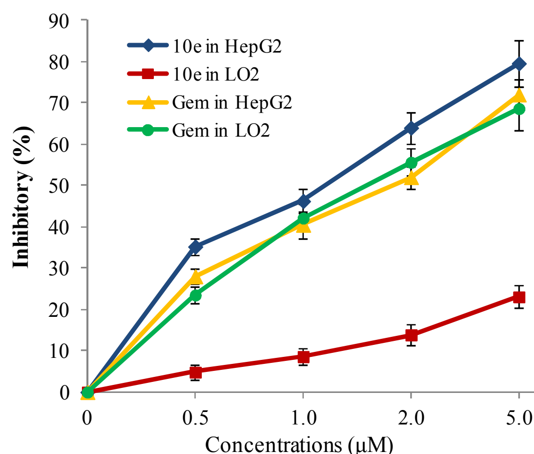

Figure 3. Inhibitory effects of **10e** on the proliferation of HepG2 and LO2 cells. Cells were incubated with the indicated concentrations of **10e** for 48 h. Cell proliferation was assessed using the MTT assay. Data are means ± SD of the inhibition (%) from three independent experiments.

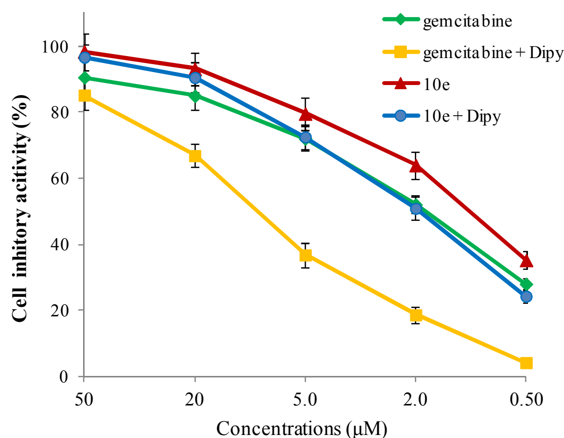

Figure 4. The effect of the nucleoside transport inhibitor dipyridamole on sensitivity to gemcitabine and **10e** was studied in HepG2 cells. HepG2 cells were treated with different concentrations (50, 20, 5.0, 2.0, and 0.50 μM) of gemcitabine and **10e** with or without dipyridamole (4 μg/mL) for 48 h. Data are expressed as means ± SD from three separate experiments.

The correction between NO production and anti-tumor activities. Furthermore, we tested whether the strong inhibition of active compounds on the proliferation of tumor cells could be associated with high levels of NO production in HepG2, HCT-116, and SW-620 cells. These cells were exposed to each compound (100 μM) for varying durations (30-300 min). The levels of

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nitrite/nitrate produced in the lysates of these cells were characterized in the Griess assay (**Figure 5A**). As expected, treatment with gemcitabine resulted in little nitrite/nitrate similar to control group in any tested cells, while treatment with hybrids (**10b-e**, **10j**, and **10k**) with high cytotoxicities produced significantly variable levels of nitrate/nitrite. In particular, **10e** exhibited the highest levels of nitrate/nitrite in these cells. These results indicate that the amounts of NO released intracellularly are closely associated with their in vitro antiproliferative activity. The NO release behaviors of **10e** along with JS-K (a known NO donor) were further examined using a NO-sensitive fluorophore, 4-amino-5-(methylamino)-2′,7′-difluorofluorescein diacetate (DAF-FM DA).27 It was observed that **10e** showed significant fluorescence in a dose-dependent manner and produced greater amounts of NO in tumor cells than JS-K (Figure 5B).

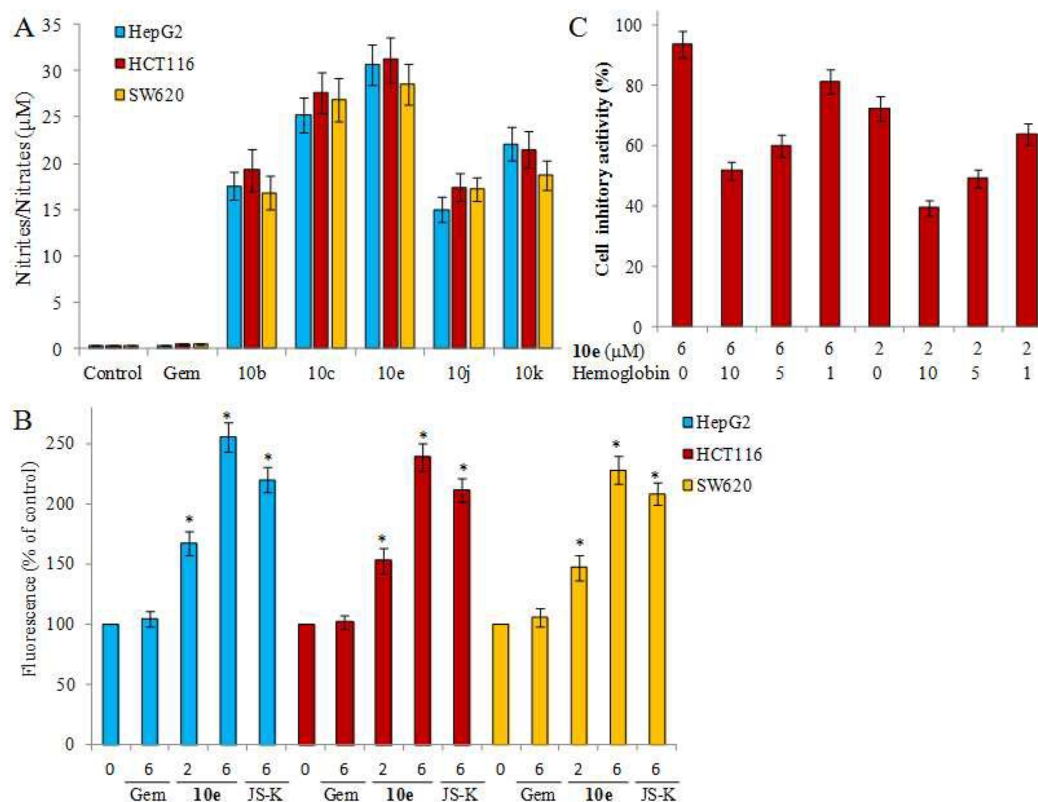

Figure 5. (A) Human cancer cells were treated with each compound at 100 μM and the contents of nitrate/nitrite in the cell lysates were determined by Griess assay through the duration of 30–300 min. The individual values were determined by measuring absorbance at 540 nm, and calculated according to the standard curve. Nitrate/nitrite production in these cells was observed at other experimental time points (data not shown). (B) DAF-FM DA (Beyotime) was used as a fluorescent indicator of intracellular NO. When cells grown in a 96-well plate reached 80% confluence, they were washed with PBS. After being loaded with 5 μM DAF-FM DA at 37 °C for 20 min, the cells were rinsed three times with PBS and incubated with test compounds for 24 h. NO production was measured with the flow cytometer with excitation and emission wavelengths of 495 and 515 nm, respectively. (C) HepG2 cells were pretreated with, or without, the indicated concentrations of hemoglobin for 1 h and treated with 2 or 6 μM of **10e** for 48 h. Data are expressed as mean% of inhibition on the growth of HepG2 cells. The cells treated with different concentrations of hemoglobin alone did not affect their growth (data not shown).

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To further determine the role of NO in cytotoxicity of these hybrids against cancer cells, we pre-treated HepG2 cells with various concentrations of hemoglobin, a well-known NO scavenger, followed by treatment with different concentrations of hybrid compound **10e** to detect their inhibitory activities and NO production (**Figure 5C**). As expected, **10e** displayed a strong anti-proliferation of HepG2 cells without pre-treatment with hemoglobin. In contrast, pre-treatment with different concentrations of hemoglobin dramatically reduced the cytotoxicity of **10e** against the HepG2 cells. Furthermore, the inhibitory effects of different concentrations of hemoglobin were dose-dependent and negatively correlated with the inhibition of the proliferation of tumor cells. Notably, treatment with 10 μM of hemoglobin reduced the cytotoxicity of **10e** by near 50%. Therefore, the high concentrations of NO produced by NO donor moieties and the bioactivity of gemcitabine synergistically contribute to the anti-tumor effects of these new hybrids in vitro.

Flow cytometry assay of cell apoptosis. To test whether the higher potency of compound **10e** is due to cell apoptosis, HepG2 cells were incubated with vehicle alone, different concentrations of **10e**, or gemcitabine, respectively, for 48 h and the percentages of apoptotic cells were determined by FITC-Annexin V/PI staining and flow cytometry. The effect of **10e** on cell apoptosis is presented in **Figure 6**. In the untreated group, the frequency of HepG2 cells apoptosis was not noticable. In contrast, the percentage of the cell apoptosis showed an upward trend in **10e**-treated HepG2 cells when the dose increased. More importantly, treatment with 2.0 μM of **10e** induced 78.3% of HepG2 cells apoptosis, which was superior to that with 2.0 μM of gemcitabine. These results demonstrated that incubation with **10e** induced HepG2 cell apoptosis in a dose-dependent manner.

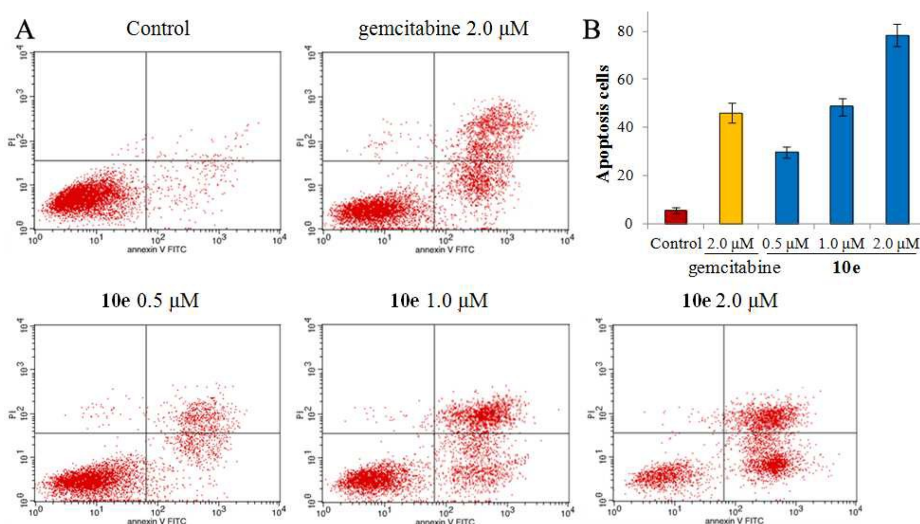

Figure 6. Compound **10e** induced HepG2 cells apoptosis in vitro. HepG2 cells were incubated with the indicated concentrations of **10e** and gemcitabine for 48 h, and the cells were stained with FITC-Annexin V/PI, followed by flow cytometry analysis. (A) Flow cytometry analysis. (B) Quantitative analysis of apoptotic cells. Data are expressed as means ± SD of the percentages of apoptotic cells from three independent experiments.

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Western blot assay. To gain insight on the underlying molecular mechanism with furoxan/gemcitabine hybrid **10e** on cell apoptosis, we conducted western blot analysis to examine the expression of apoptosis proteins in HepG2 cells. It is well known that Bcl-2 and Bax are anti-apoptotic or pro-apoptotic regulator proteins, respectively, and caspase-3 is the execution factor of apoptosis. In addition, Parp-1 is one of several known cellular substrates for caspase-3 and cleavage of Parp-1 by caspase-3 is considered to be a hallmark of apoptosis.[23] As shown in **Figure 7**, the protein level of Bcl-2 was dramatically reduced and the expression of Bax proteins was significantly increased in **10e-**treated cells in a dose-dependent manner. Moreover, the level of activated caspase-3 was greatly enhanced, and the decreased expression of Parp-1 in **10e-**treated cells was also found which was greater than that treated with 2.0 μM of gemcitabine. These changes indicated that the **10e** could significantly induce cell apoptosis through the regulation of apoptosis proteins.

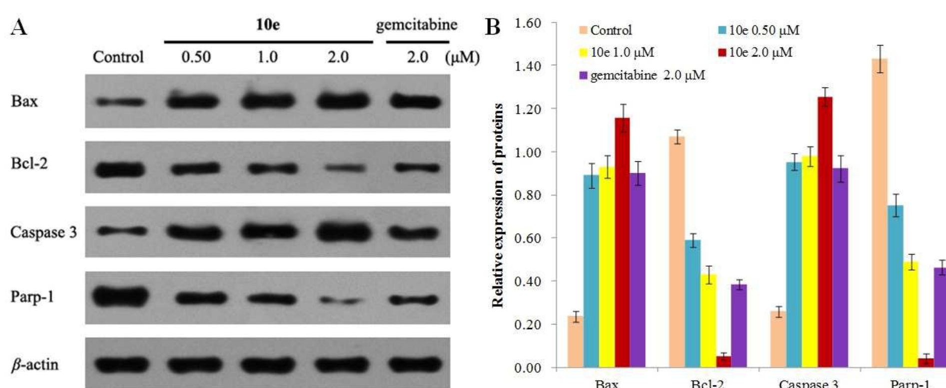

Figure 7. Effect of compound **10e** on the expression of apoptosis-related proteins in HepG2 cells. (A) The expression of Bax, Bcl2, caspase 3, Parp-1, and β-actin were examined by western blot analysis. HepG2 cells were incubated with or without **10e**, or gemcitabine at the indicated concentrations for 48 h and the levels of protein expression were detected using specific antibodies. Data shown are representative images of each protein for three separate experiments. (B) Quantitative analysis: the relative levels of each protein compared to the control β-actin were determined by densimetric scanning. Data are expressed as means ± SD from three separate experiments.

Analysis of SAR revealed that most of **10a-m** exhibited remarkable anti-tumor activities against five human cancer cells. It was discovered that the length of the linker and linker type have significant influence on the inhibitory activities against the cancer cell growth. For example, compounds **10a-h** with diol linker chain showed relatively higher antiproliferative activities compared to compounds **10i-m** with alcohol amine chain. As for the length of the linker, hybrids (**10b-e**, **10j**, and **10k**) with the linker comprised of three or four carbon units displayed considerably greater growth inhibitory effects than the other hybrids. However, some compounds with aryl or ether bond chain were less effective than those with the aliphatic linker chain. In sharp contrast, among tested compounds, the hybrid **10e** with 1,4-butyl acetylene glycol group had the

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strongest inhibitory activity, which may result from the synergic effects of the NO donor moiety and gemcitabine moiety.

Conclusion

In summary, a series of novel nitric oxide (NO)-releasing hybrids **10a-m** from gemcitabine and phenylsulfonyl furoxans were designed and synthesized. Most hybrids possessed potent antiproliferative activities against five human cancer cell lines in vitro. In particular, compound **10e** not only exhibited the strongest anti-tumor activity, but also produced high levels of NO. Furthermore, inhibition of nucleoside transport significantly reduced the inhibitory activity of gemcitabine, but not **10e**, suggesting the independence of a nucleoside transporter. More importantly, treatment with hemoglobin partially reduced cytotoxicities of **10e** in HepG2 cells. These results suggested that the high concentration of NO release by NO donor moieties of **10e** and the bioactivity of gemcitabine segment may synergistically contribute to the anti-tumor activity of **10e** in vitro. In addition, **10e** induced cancer cells apoptosis by reducing the levels of Bcl-2 and Parp-1 and up-regulating the expression of Bax and caspase-3. Our findings suggest that **10e** may hold promise as therapeutic agents for the intervention of human cancers.

Acknowledgments

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Synthesis and biological evaluation of nitric oxide-releasing hybrids from gemcitabine and phenylsulfonyl furoxans as anti-tumor agents

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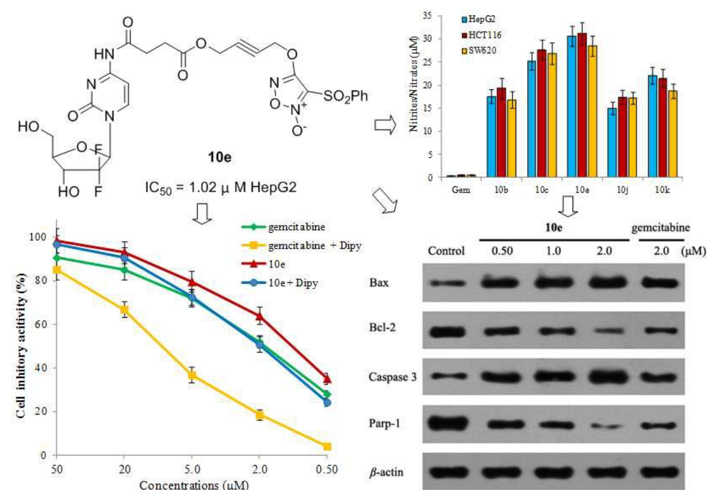
Graph Abstract



Novel furoxan/gemcitabine hybrids displayed significant antitumor activities, in particular **10 e**, which could be independent to the nucleoside transporter, release high levels of NO, and induce cell apoptosis by regulating apoptotic related proteins in tumor cells *in vitro*.

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