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Novel anticancer oridonin derivatives possessing a diazen-1-ium-1,2-diolate nitric oxide donor moiety: Design, synthesis, biological evaluation and nitric oxide release studies

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ABSTRACT

Oridonin (**1**) is a complex *ent*-kaurane diterpenoid with unique antitumor profile, which is isolated from *Isodon rubescens*. In order to develop novel derivatives of oridonin with improved potency, a series of nitric oxide (NO)-releasing oridonin derivatives were synthesized by coupling diazeniumdiolates with oridonin and its semisynthesized analogues. Their in vitro antiproliferative activity, nitric oxide release ability, and preliminary anticancer mechanism were further evaluated. The results displayed that all the target compounds exhibited potent antiproliferative activities, with IC_{50} values ranging from 1.84 to 17.01 μM. Besides, it was observed that in most cases, the antiproliferative activity correlated well with levels of intracellular NO release. More interestingly, preliminary mechanism studies revealed that the most potent compound **14d** induced apoptosis and arrested the cell cycle at the S phase in Bel-7402 cells, which is different from parent compound oridonin. Together, the above promising results warrant the further development of oridonin/NO hybrids as potential antitumor leads.

In the past few decades, a worldwide increase in the incidence of malignancy has been observed and cancer has become the second leading cause of death in the world, after heart disease.[1] This has led to the search for new antineoplastic agents, particularly those obtained from natural sources such as plants, insects, and marine microorganisms.[2] Over the past century, natural products have provided a major source of therapeutic agents for human diseases[3,4], especially in recent years, a large number of terpenes has marked anticancer effects toward various types of cancer cell lines in vitro, some of them have been successfully developed for clinical use to treat human neoplastic diseases.[5]

Isodon species are widely distributed plants, many of which have been used extensively in folk medicine.[6] Oridonin (**1**) is a complex *ent*-kaurane diterpenoid isolated from *Isodon rubescens*, which has been attracting a rising attention in recent years from cancer biologists due to its attractive antitumor activity[7–10] (Fig. 1). Its unique skeleton as well as potent antitumor activity makes it promising candidate as an antitumor agent. However, the development of oridonin for cancer therapy was hampered largely by its moderate activity and structural complexity. Therefore, it is highly desirable to develop novel derivatives of oridonin to improve its potency without reducing its safety profile.

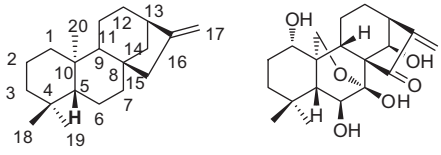

Figure 1. Structures of *ent*-kaurane diterpenoid and oridonin.

Nitric oxide (NO) is an important signal molecule involved in regulating the numerous physiologic and pathologic processes.[11] Various studies have shown that high levels of NO can inhibit the growth of tumor cells and promote their apoptosis through many mechanisms.[12] Due to the limited utility of NO gas, nitric oxide donors are typically used as surrogates for NO in anticancer studies.[13] Among the available NO donors, diazeniumdiolates (NONOates) are used in a number of biological studies as reliable

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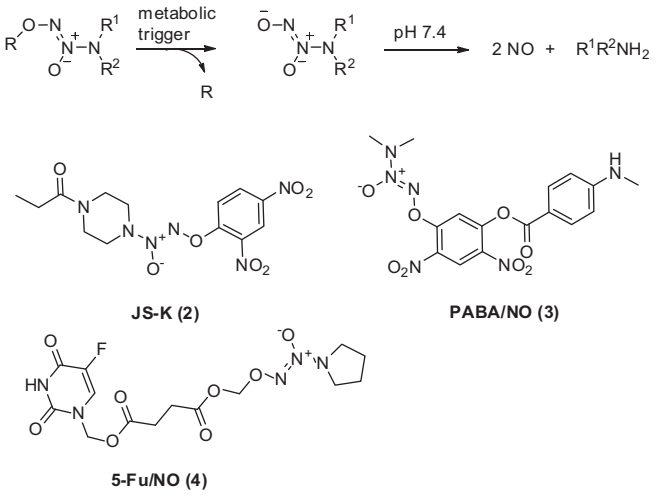


Figure 2. Activation of diazeniumdiolate prodrugs to release NO and structures of JS-K (**2**), PABA/NO (**3**), 5-Fu/NO (**4**).

sources of NO.[14] As shown in Figure 2, diazeniumdiolate prodrugs would form the parent anion under physiological conditions (pH 7.4, 37 °C), which further decomposes to release up two moles of NO. This excellent performance makes them especially attractive for designing antitumor drugs. Recently, a variety of promising diazeniumdiolate-based NO-releasing derivatives have been developed as anticancer agents, such as JS-K (**2**), PABA/NO (**3**), and 5-Fu/NO (**4**). JS-K releases NO on reaction with glutathione (GSH), and has proven effective in several in vitro and in vivo models.[15] PABA/NO has been shown to have anticancer activity comparable to that of cisplatin in human ovarian cancer xenograft studies.[16] The 5-Fu/NO conjugate synthesized by Cai et al. showed greater cytotoxicity than 5-Fu against DU145 human prostate and HeLa cancer cells[17] (Fig. 3).

Enlightened by these findings, together with our previous studies on the modification of oridonin[18], as a part of our ongoing program, we designed and synthesized a series of novel oridonin hybrides containing diazeniumdiolates to improve the antiproliferative activity of natural oridonin. Hererin, we report the synthesis, in vitro antiproliferative activity, NO release, and anticancer mechanism for a new class of NO-releasing oridonin hybrides.

As illustrated in Scheme 1, the synthesis of NONOates was carried out by our previously reported procedure in the presence of nanometre-sized TiO_2.[19] In general, preparation of NONOates requires considerably strict conditions, such as special apparatus, low temperature and high pressure.[20] In our developed novel preparative approach of NONOates through nanometre-sized TiO_2, reaction of aliphatic cyclic amines with nitric oxide gas at room temperature under atmospheric pressure afforded O^2-sodium-1-(alkylamino)diazen-1-ium-1,2-diolates (**6**) in high yield. The sodium salts were alkylated with chloromethyl methyl sulfide to afford O^2-(methylthiomethyl)-1-(alkylamino)diazen-1-ium-1,2-diolates (**7**), which were subsequently reacted with sulfuryl chloride in dichloromethane to afford the O^2-chloromethyl-protected diazeniumdiolates (**8**). This preparative approach of NONOates under atmospheric pressure using easily available apparatus promoted the following synthesise of oridonin/NONOate hybrides.

The target oridonin/NONOate hybrides **13a–d**, **14a–d**, and **15a** were synthesized in moderate yields as shown in Scheme 2 by condensation of oridonin and its semisynthesized analogues (**11**, **12**) with O^2-chloromethyl intermediates **8a** or **8b** using diverse diacids as a linker. Treatment of **1** with Jones reagent at 0 °C afforded corresponding ketone **12** in yield of 94%. Reaction of **1** with 2,2-dimethoxypropane in the presence of TsOH in acetone provided

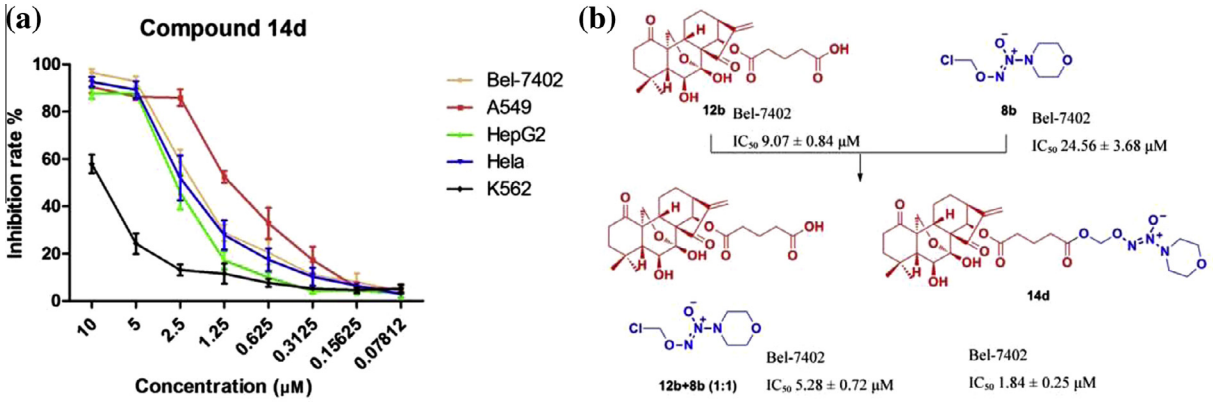
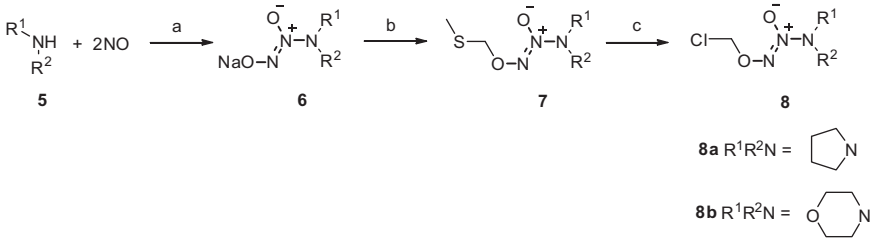
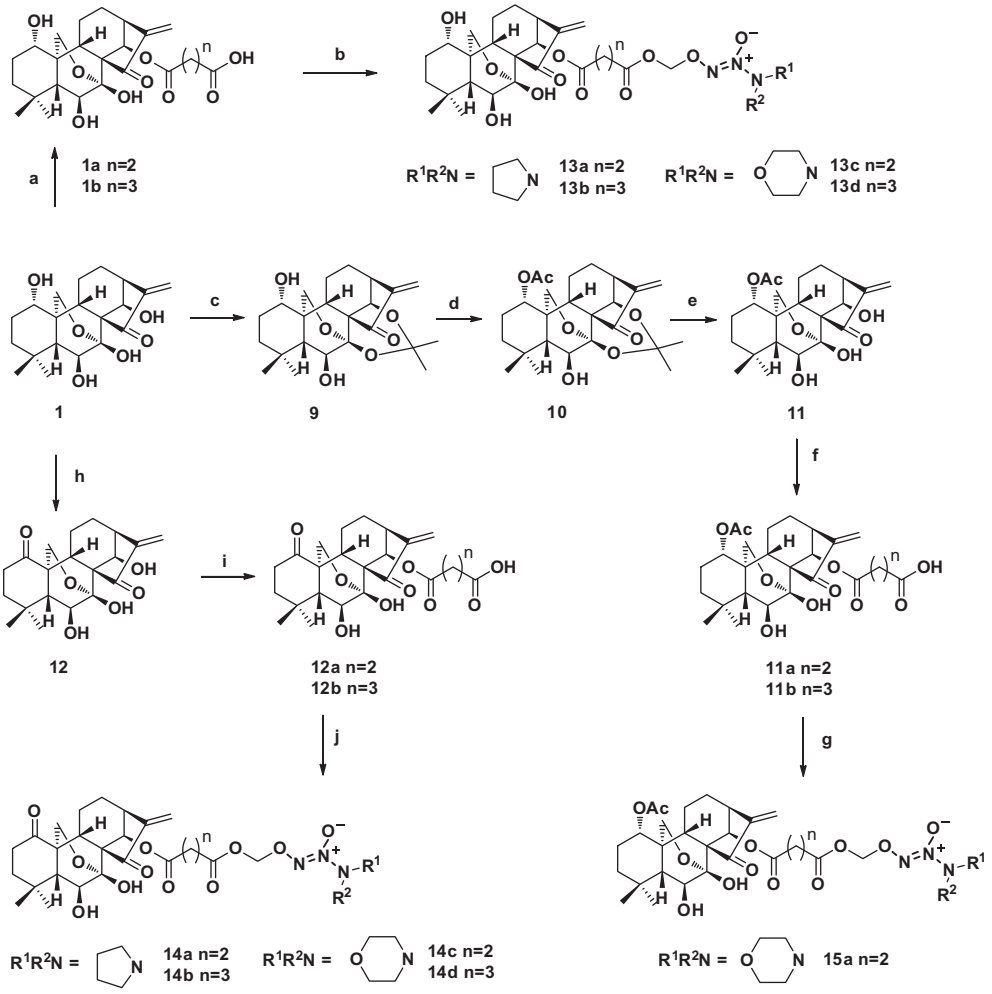


Figure 3. (a) Compound **14d** inhibits cells proliferation in various cancer cell lines. Kinds of cancer cells were treated with different concentrations of **14d** for 72 h, IC_{50} values for each cell lines determined using MTT assay are as follows: Bel-7402 = 1.84 μM, A549 = 1.13 μM, HepG2 = 2.57 μM, Hela = 2.46 μM, K562 = 8.48 μM; (b) comparison of antiproliferative activity of hybrid **14d** with its components **12b**, **8b**, and the equimolar mixture of **12b** and **8b**.



Scheme 1. Synthesis of NONOates through nanometre-sized TiO_2-catalyzed reactions of NO with aliphatic cyclic amines. Reagents and conditions: (a) MeONa, MeOH, nanometre-sized TiO_2, rt, 48 h; (b) $ClCH_2SCH_3$, DMF, Na_2CO_3, rt, 48 h; (c) SO_2Cl_2, DCM, rt, 3 h.



Scheme 2. General synthetic scheme for NONOates-oridonin hybrides (**13a–c**, **14a–c**, **15a**). Reagents and conditions: (a) Anhydrides, DCM, DMAP, rt, 36–48 h, 76–83%; (b) Cs_2CO_3, DMF, rt, 6 h, 12–46%; (c) DMP, TsOH, Acetone, 70 °C, 10 min, 97%; (d) Ac_2O, DMAP, TEA, DCM, rt, 3 h, 94%; (e) 10% HCl, THF, 0.5 h, 97%; (f) Anhydrides, DCM, DMAP, rt, 36–48 h, 76–83%; (g) Cs_2CO_3, DMF, rt, 6 h, 12–46%; (h) Jones reagents, Acetone, 0 °C, 10 min, 94%; (i) Anhydrides, DCM, DMAP, rt, 36–48 h, 76–83%; (j) Cs_2CO_3, DMF, rt, 6 h, 12–46%.

Table 1
Growth inhibitory effects of target compounds on human hepatocellular carcinoma Bel-7402 cells[a]

Compounds	Inhibition rate % (Bel-7402)	
	1 μM (%)	10 μM (%)
1	29.91	93.63
13a	15.93	93.22
13b	26.10	93.37
13c	88.45	93.26
13d	83.85	92.73
14a	24.17	93.58
14b	13.46	93.94
14c	71.33	93.11
14d	87.77	93.94
15a	85.82	94.45

[a] Bel-7402 cells were treated with different concentrations (1, 10 μM) of indicated compounds for 72 h. Values are the mean of three independent experiments.

ketal **9** in 97% yield; compound **9** upon reaction with Ac_2O/DMAP/TEA led to acetylated compound **10** in the yield of 94%; deprotection of **10** with 10% HCl gave the corresponding alcohol **11** in almost quantitative yield. Subsequent reaction of these analogues or oridonin with anhydrides afforded acids (**1a–b**, **11a–b**, **12a–b**) for the final condensation reaction.

We evaluated the preliminary inhibitory effects of oridonin (**1**) and its NO-based derivatives (**13a–d**, **14a–d**, **15a**) on cell growth by an MTT method using human hepatocellular carcinoma Bel-7402 cells. As shown in Table 1, all these newly synthesized hybrides displayed comparable or even higher inhibition rate than oridonin at a concentration of 1 μM, these results inspired us for the further studies of these compounds on anticancer effects. As shown in Table 2, all the target compounds were found to possess potent antiproliferative activity, with IC_{50} values ranging from 1.84 to 17.01 μM and in most cases better than that of oridonin. The most potent compound **14d**, whose IC_{50} value was 1.84 μM against Bel-7402 cells, were further tested for its antiproliferative activity

Table 2
Antiproliferative activity of the target compounds against Bel-7402 cells[a]

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
Adriamycin	0.83 ± 0.12	**14a**	14.58 ± 1.35
1	7.12 ± 0.64	**14b**	17.01 ± 1.93
13a	13.27 ± 1.32	**14c**	3.26 ± 0.44
13b	10.94 ± 0.84	**14d**	1.84 ± 0.25
13c	2.75 ± 0.18	**15a**	3.03 ± 0.40
13d	2.53 ± 0.29		

[a] MTT methods, cells were incubated with indicated compounds for 72 h, (means ± SD, $n = 3$).

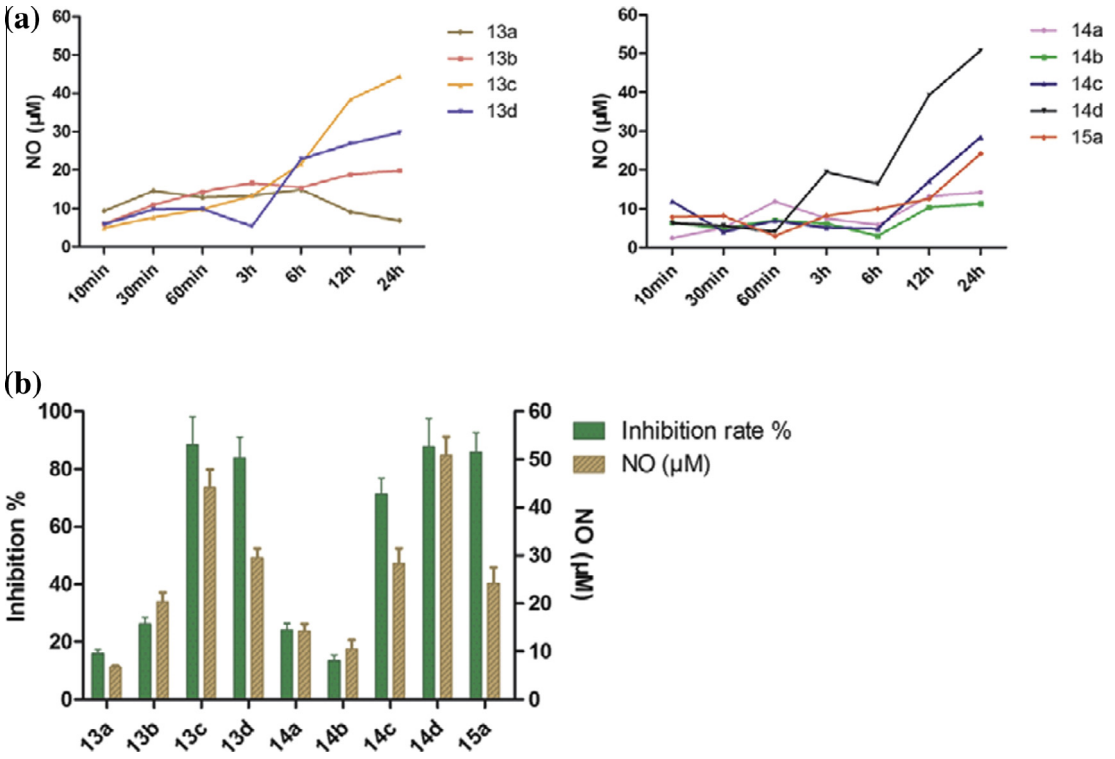


Figure 4. The concentrations of NO produced by target compounds were correlated well with their antiproliferative activities against Bel-7402 cells. (A) Variable levels of NO produced by target compounds in Bel-7402 cells. Cells were cultured at a density of 15,000/mL, then treated in triplicate with each compound at 50 μM for different time, and then the contents of nitrate/nitrite in the cell lysates were determined by the Griess assay. Values are the mean of three independent experiments. (B) Antiproliferative activity were expressed as inhibition rate at 1 μM, and the NO productions were determined after 6 h incubation.

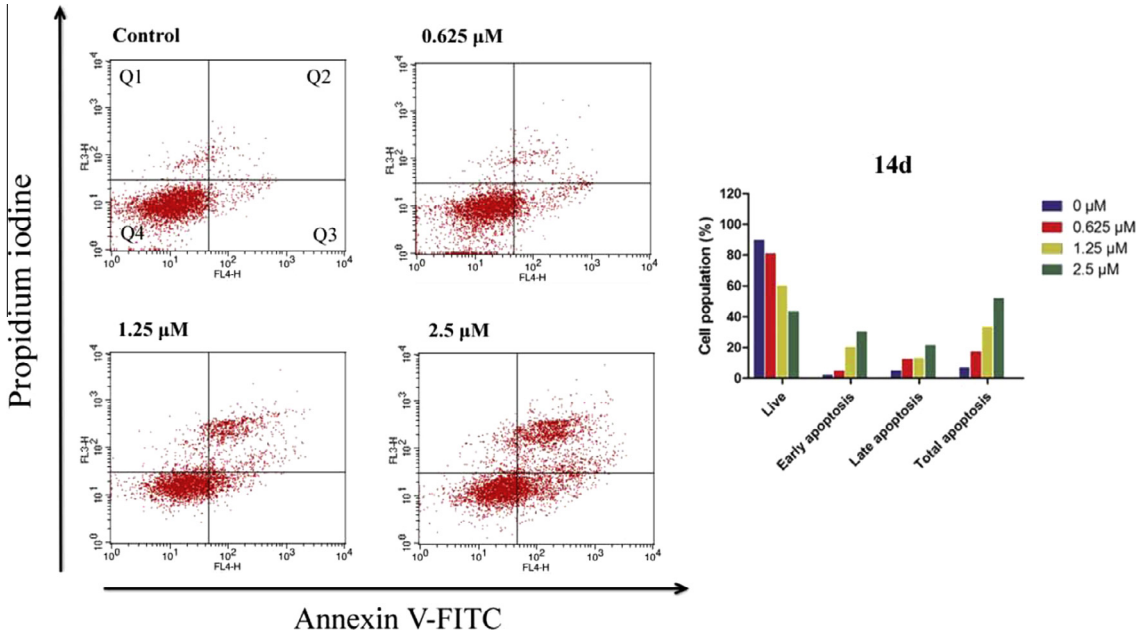


Figure 5. Effect of **14d** on the induction of apoptosis in Bel-7402 cells. After 36 h of **14d** treatment (0, 0.625, 1.25, 2.5 μM), Bel-7402 cells were collected and stained with Annexin V/PI, followed by flow cytometric analysis, histogram displays the percentage of cell cycle distribution.

against a number of other human cancer cell lines, including A549, HepG2, Hela, and K562 cells. The results showed that **14d** was generally potent in all tested cell lines and the A549 cell appeared to be the most sensitive cell line (Fig. 3a). Since **14d** is composed of two moieties, oridonin analogue (compound **12b**) and NONOate (**8b**), thus, the additive effects of their antiproliferative activity in Bel-7402 cells were further examined. As shown in Figure 3b, the IC_{50} value of **14d** was significantly less than that of individual moieties **12b** and **8b**, and even their combination. These results suggested that the antiproliferative activity of **14d** could be attributed to the synergic effects of oridonin and NO donor moiety in cancer cells.

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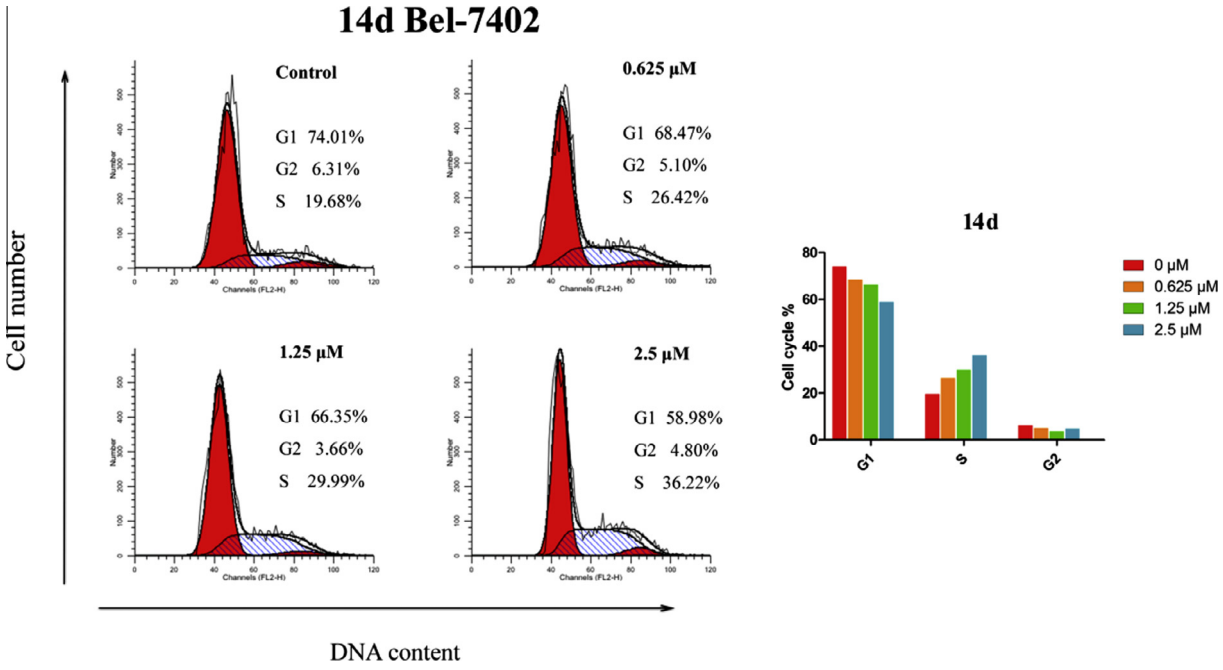

Figure 6. Compound **14d** induces S-phase arrest in Bel-7402 cells. Cells were treated with DMSO and varying concentrations of **14d** (0, 0.625, 1.25, 2.5 μM) for 36 h and satined with PI. The cell cycle distributions were analyzed by flow cytometry, histogram displays the percentage of cell cycle distribution.

It is important to identify and quantitate the NO released on activation of these hybrides in cancer cells to assess whether the level of NO production was associated with their cytotoxicities. Nitrate and nitrite, the oxidative end products of NO, were measured in the supernatants by means of the Griess assay to quantitate the released NO. As shown in Figure 4, abundant NO was released from most of these hybrides, and treatment with **13c** and **14d** generated higher concentrations of nitrite/nitrate production. Furthermore, it was observed that in most cases, the antiproliferative activities correlated well with levels of intracellular NO release. The analogues that released higher amounts of NO had superior antiproliferative activity (**13c** and **14d**), while the analogues that produced minor amounts of NO were less active against Bel-7402 cells (**13a** and **14b**), suggesting that nitric oxide is a key factor in the cytotoxicity of such hybrides.

Previous studies have shown that oridonin could induce cancer cell apoptosis by modulating the expression of apoptosis regulators.[21] Besides, high levels of NO can induce tumor cell apoptosis.[22] Therefore, the effect of **14d** on induction of tumor cell apoptosis was further tested by flow cytometry analysis via Annexin V/PI staining. Bel-7402 cells were treated with variable concentrations of **14d** for 36 h, the cells were harvested and stained with Annexin V-FITC and propidium iodide (PI). Annexin V is a phospholipid-binding protein with strong affinity for phosphatidylserine, which appears on the cell surface as a general indicator of apoptosis. As shown in Figure 5, the percentage of Annexin V-positive cells (right quadrants, Q2 + Q3) increased to 51.91% after treatment with 2.5 μM of **14d**, compared to only 6.20% of the control cells.

To obtain further insight into the mechanism of **14d** in cell cycle arrest, Bel-7402 cells were incubated with varying concentrations of **14d** for 36 h, stained with PI, and analyzed by flow cytometry. Indeed, **14d** treatment lead to a dose-dependent induction of cell cycle arrest in the S-phase (Fig. 6). When Bel-7402 cells were treated with different concentrations of compound **14d**, the percentage of cells in S fraction increased from 19.68% to 36.23%, while the percentage of cells in G1 phase decreased from 74.01% to 58.98%, which is different from parent compound oridonin.

Conclusions

In this effort, a number of oridonin/NONOate hybrides were synthesized and their biological functions were evaluated. Most compounds were found to have excellent antiproliferative ability in human leukemia Bel-7402 cells. Notably, the most potent compound **14d** exhibited good inhibitory activity on a panel of human cancer cell lines. NO release assay indicated that the potent antiproliferative activities of tested compounds were associated with high levels of NO produced from diazeniumdiolates, and the synergic effects of oridonin and NO donor moiety. Importantly, the mechanism of action (MOA) of the promising compound **14d** refers to the induction of apoptosis and cell cycle arrest at S-phase. Together, these findings provide a new insight for the rational design of NO-based oridonin derivatives to enhance its anticancer efficacy and may open a possible avenue to the development of NO-based hybrids as potential anticancer agents.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.068.

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