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Novel Hybrids of Natural Oridonin-Bearing Nitrogen Mustards as Potential Anticancer Drug Candidates

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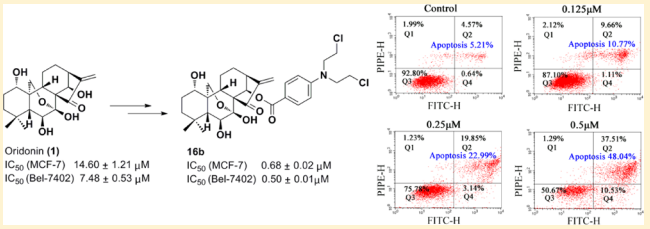
Supporting Information

ABSTRACT: A series of novel hybrids from natural product oridonin and nitrogen mustards were designed and synthesized to obtain more efficacious and less toxic antitumor agents. The antiproliferative evaluation showed that most conjugates were more potent than their parent compounds oridonin and clinically used nitrogen mustards against four human cancer cell lines (K562, MCF-7, Bel-7402, and MGC-803). Furthermore, the representative compounds **16a−c** exhibited antiproliferative activities against the multidrug resistant cell lines (SW620/AD300 and NCI-H460/MX20). It was shown that the most effective compound **16b** possesses a strong inhibitory activity with an IC_{50} value 21-fold lower than that of oridonin in MCF-7 cells and also exhibits selective cytotoxicity toward the cancer cells. Intriguingly, compound **16b** has been demonstrated to significantly induce apoptosis and affect cell cycle progression in human hepatoma Bel-7402 cells.



KEYWORDS: *Oridonin, antiproliferative activities, nitrogen mustards, combination principle, drug-resistant, apoptosis*

Cancer is a major global health problem, representing the second-leading cause of death worldwide.[1] Effective anticancer agents with novel scaffolds or new mechanisms of action are urgently needed for the highly aggressive and drug-resistant cancer.

Nitrogen mustards are among the DNA alkylating agents that are most widely used in cancer chemotherapy.[2] However, the lack of drug-specific affinity toward cancer cells necessitates the use of a large dose of a drug to achieve high local concentration, systemic toxicity leading to many side effects, and the failure of chemotherapy due to acquired drug resistance limited the use of nitrogen mustards in clinics.[3] In this regard, it is necessary to carry out chemical modifications on the nitrogen mustards to acquire new agents with enhanced anticancer activity, reduced systemic toxicity, and high selectivity for chemotherapy of cancer.[4]

Combination principle is used widely in drug design.[5] There have been a great deal of reports describing the hybrids between natural products and anticancer drugs,[6] in which the individual molecules are commonly connected via a chemically stable linker.[7] For example, prednimustine (Figure 1), the prednisolone ester of chlorambucil, not only exhibits a similar profile to that of chlorambucil in murine tumors[8] but also demonstrates activity against chlorambucil-resistant tumors.

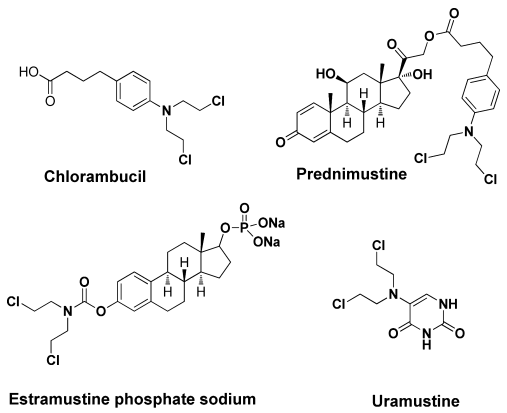


Figure 1. Structures of chlorambucil and available anticancer drugs designed by using combination principle.

Also included within this group are estramustine phosphate sodium and uramustine (Figure 1).[9]

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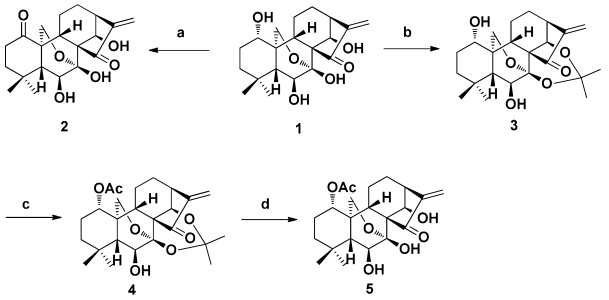
Natural diterpenoids with unique chemical skeletons and interesting activities have attracted our attention and aroused our curiosity for intensive study. Oridonin (**1**) is a widely distributed *ent*-kaurene in the *Rabdosia* plants and has recently attracted much attention due to its extensive biological activities.[10] Although oridonin exhibited unique, safe, broad antitumor activity, the development of oridonin for cancer therapy was hampered by its relatively moderate potency. Therefore, developing novel oridonin derivatives by rational modification to improve its anticancer activity profile is in urgent need.[11,12]

In our previous studies, we disclosed that some novel 1-*O*- and 14-*O*-derivatives of oridonin showed excellent cytotoxicities against several human cancer cell lines *in vitro* and *in vivo*. It was found that the modification on the 14-hydroxy of oridonin did not diminish the cytotoxicities against a panel of human cancer cell lines.[13] These findings prompted us to introduce different kinds of nitrogen mustards to the 14-hydroxy of oridonin, including chlorambucil, melphalan, and formylmerphalan. In addition, the benzoic acid mustard was also chosen due to its low toxicity.[14,15]

Herein, in this presentation, we describe the design and synthesis of a series of novel oridonin-coupled nitrogen mustard conjugates. It is hoped that the oridonin/nitrogen mustard hybrids could exhibit potent synergistic anticancer effect, leading to a higher antiproliferative efficacy, a broader therapeutic scope, and a lower systemic toxicity.

First, the oridonin analogues (**2** and **5**) were synthesized as shown in Scheme 1 according to our previous protocols.[16] The

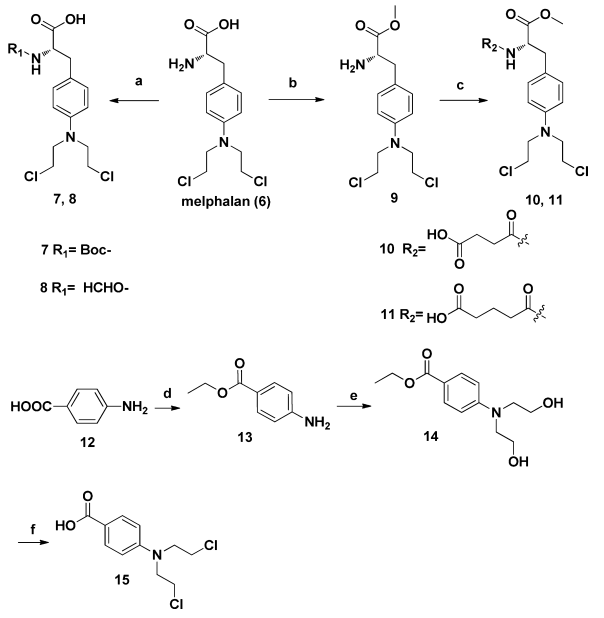
Scheme 1. Synthesis of Oridonin Analogues 2 and 5[a]



[a]Reagents and conditions: (a) Jones reagent, acetone, 0 °C, 10 min, 88%; (b) 2,2-dimethoxypropane, acetone, TsOH, 56 °C, 20 min, 85%; (c) Ac_2O, TEA, DMAP, rt, 2 h, 95%; (d) 10% HCl, THF (1:1), rt, 30 min, 98%.

oxidation of **1** with Jones reagent afforded ketone **2**. The treatment of **1** with 2,2-dimethoxypropane in the presence of TsOH provided ketal **3** in 85% yield. Compound **3** upon reaction with Ac_2O/DMAP/TEA led to acetylated compound **4** in the yield of 95%. Deprotection of **4** gave the corresponding alcohol **5** in almost quantitative yield. Then the nitrogen mustard intermediates were prepared as depicted in Scheme 2. The selective protection of the primary amine of melphalan (**6**) afforded Boc-protected melphalan (**7**) or formylmerphalan (**8**). Esterification of **6** carried out in dry methanol in the presence of $SOCl_2$ led to ester **9** in quantitative yield, and subsequent reaction of **9** with succinic anhydride or glutaric anhydride provided melphalan derivatives **10** and **11**, respectively. The benzoic acid mustard (**15**) was prepared according to the literature procedure.[14] At last, the target oridonin-coupled

Scheme 2. Synthesis of Melphalan Derivatives 7, 8, 10, and 11 and Benzoic Acid Mustard 15[a]



[a]Reagents and conditions: (a) Boc_2O, TEA, dioxane, rt, 3 h, 95% for compound **7**; Ac_2O, HCOOH, 50 °C, 5 h, 67% for compound **8**; (b) $SOCl_2$, MeOH, reflux, 12 h, 93%; (c) corresponding anhydrides, DMAP, DCM, rt, 18 h, 93% for compound **10**; 24 h, 91% for compound **11**; (d) ethanol, H_2SO_4, reflux, 5 h, 94%; (e) ethylene oxide, H_2O, HCOOH, rt, 24 h, 81%; (f) (i) $POCl_3$, 50 °C, 0.5 h; (ii) 10% HCl, 12 h, 81% over two steps.

nitrogen mustard conjugates (**16a−f**, **17a−c**, and **18a−c**) were obtained through the synthetic route outlined in Scheme 3.

The oridonin-coupled nitrogen mustard derivatives were screened for their *in vitro* antiproliferative activities against four human cancer cell lines, human leukemia K562 cells, breast adenocarcinoma MCF-7 cells, human hepatocellular carcinoma Bel-7402 cells, and MCG-803 human gastric cancer cells, by the standard MTT methods, and the results are summarized in Table 1. The IC_{50} values revealed that all the target compounds were more potent than positive control drug chlorambucil and melphalan whatever the cell line considered, and most of the conjugates exhibited more potent inhibitory activities against four cancer cell lines than their parent oridonin. As shown in Table 1, although melphalan showed more potent cytotoxicity than chlorambucil and benzoic acid mustard, compounds **16a** and **16d−f** incorporated oridonin with melphalan showed less activity than compounds **16b−c** that are incorporated with chlorambucil or benzoic acid mustard. Considering compounds **16a**, **17a**, and **18a**, varying only the substitution on C-1 position of oridonin, the IC_{50} values of **16a** were higher than those of **17a** and **18a** in all tested cell lines. However, when conjugated with chlorambucil or benzoic acid mustard, the modification on the C-1 position of oridonin had no impact on the cytotoxicities of these conjugates; **16b−c**, **17b−c**, and **18b−c** all had relatively high antiproliferative activities against four cancer cell lines. It is interesting that the benzoic acid mustard has less cytotoxicity, while when incorporated with oridonin, conjugate **16b** with OH at C-1, is the most potent hybrid among all the target compounds, with the IC_{50} values at 1.12 ± 0.07, 0.68 ± 0.02, 0.50 ± 0.01, and 1.09 ± 0.14 μM for K562, MCF-7, Bel-7402, and MGC-803, respectively.

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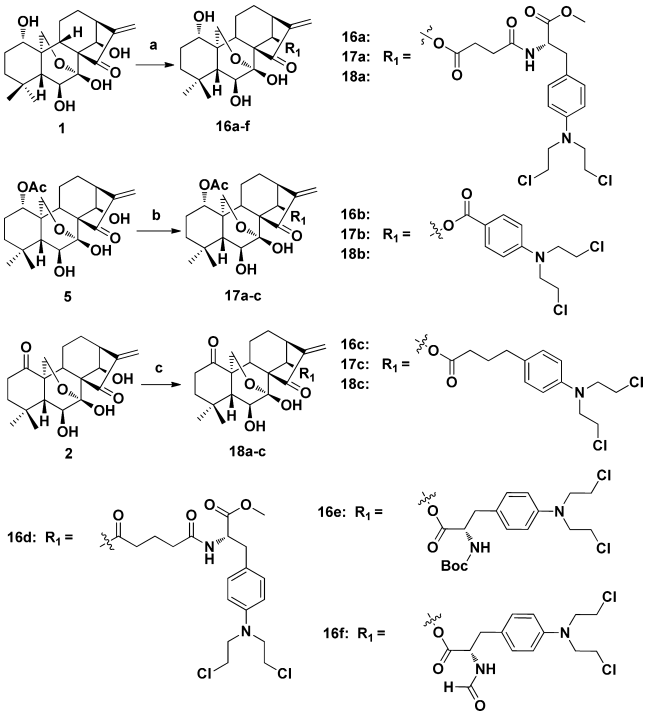
Scheme 3. Synthesis of Oridonin Nitrogen Mustard Derivatives 16a−f, 17a−c, and 18a−c[a]



[a]Reagents and conditions: (a) **7**, **8**, **10**, **11**, **15**, chlorambucil, EDCI, DMAP, DCM, rt, 12−24 h, 60%−82%; (b) **10**, **15**, chlorambucil, EDCI, DMAP, DCM, rt, 9−16 h, 71%−83%; (c) **10**, **15**, chlorambucil, EDCI, DMAP, DCM, rt, 12−18 h, 72−78%.

Multidrug resistance (MDR) is one of the major reasons for the failure of cancer chemotherapy.[17] One of the major mechanisms in cancer cells that give rise to MDR is the overexpression of ATP-binding cassette (ABC) transporters.[18] To investigate whether these nitrogen mustard-fused oridonin derivatives are effective on drug-resistant ABCB1-overexpressing SW620/AD300 and ABCG2-overexpressing NCI-H460/MX20 cells, representative compounds **16a−c**, which are coupled with melphalan, benzoic acid mustard, and chlorambucil, respectively, were selected to test their antiproliferative activities against the drug-resistant and parental sensitive cells by using MTT methods. As shown in Table 2, oridonin had moderate anticancer activities against both sensitive and drug-resistant cells. Unfortunately, when conjugated with melphalan, the antiproliferative activity of compound **16a** decreased, especially on the NCI-H460/MX20 cells, which did not exhibit any cytotoxicity even at a concentration of 100 μM. This result indicated that **16a** may be a substrate of multidrug transporter BCRP (breast cancer resistance protein, also called ABCG2). To our delight, compounds **16b** and **16c**, the 14-OH modified oridonin ester of benzoic acid mustard or chlorambucil, not only displayed potent antiproliferative activities against cancer cells but also demonstrated activities against drug-resistant cancer cells. Particularly, hybrid **16b** exhibited the most potent antiproliferative activities against SW620, SW620/AD300, NCI-H460, and NCI-H460/MX20 cells with IC_{50} values at 1.96 ± 0.11, 1.86 ± 0.06, 2.35 ± 0.14, and 2.91 ± 0.12 μM, respectively. Therefore, it clearly illustrated that multidrug transport P-glycoprotein (P-gp/ABCB1) did not affect antiproliferative activities of these compounds. As a result, the design of hybrids from natural product and nitrogen mustards appears to be a viable strategy to address the problem of MDR in cancer therapy.

Nonselective cytotoxicity is also the main effect that limits the use of optimal doses in most conventional chemotherapeutic regiments.[19] In an attempt to estimate the toxicity of conjugates **16b** and **16c** compared with chlorambucil or benzoic acid mustard, we tested the toxicities of the compounds **1**, **15**, **16b**, **16c**, and chlorambucil on the human normal liver cells L-02 and human liver cancer cells Bel-7402. Cell apoptosis induced by these compounds is both concentration and time dependent; the survival curves of **16b** and **16c** in Bel-7402 and L-02 cells after a 72 h treatment showed that 90.10% kill was achieved for **16b** in Bel-7402 at the concentration of 2.5 μM, but only 25.1% human normal liver L-02 cells were killed at this concentration. For compound **16c**, 90.4% Bel-7402 cells were

Table 1. IC_{50}[a] Values (μM) of Synthetic Oridonin Nitrogen Mustard Derivatives (16a−f, 17a−c, and 18a−c) against Human Cancer Cell Lines[b]

compd	K562	MCF-7	Bel-7402	MGC-803
oridonin	4.76 ± 0.32	14.60 ± 1.21	7.48 ± 0.53	5.69 ± 0.37
chlorambucil	60.12 ± 5.48	80.54 ± 4.73	49.31 ± 2.87	133.64 ± 11.25
melphalan	33.37 ± 1.16	19.58 ± 1.02	24.76 ± 1.35	36.88 ± 1.21
benzoic acid mustard (**15**)	142.67 ± 10.23	152.15 ± 8.66	>200	146.20 ± 9.73
16a	9.01 ± 0.78	14.37 ± 1.24	8.35 ± 0.74	9.30 ± 0.18
16b	1.12 ± 0.07[c]	0.68 ± 0.02[c]	0.50 ± 0.01[c]	1.09 ± 0.14[c]
16c	2.28 ± 0.69[c]	1.32 ± 0.05[c]	1.43 ± 0.09[c]	2.02 ± 0.27[c]
16d	5.74 ± 0.45	12.90 ± 0.96	7.91 ± 0.85	6.92 ± 0.45
16e	1.41 ± 0.10[c]	3.22 ± 0.45[c]	2.36 ± 0.67[c]	6.72 ± 0.58
16f	7.03 ± 0.34	14.58 ± 0.28	3.84 ± 0.32[c]	7.10 ± 0.33
17a	1.73 ± 0.09[c]	3.63 ± 0.15[c]	2.56 ± 0.51[c]	4.71 ± 0.16
17b	1.35 ± 0.12[c]	0.91 ± 0.03[c]	2.41 ± 0.23[c]	3.76 ± 0.53[c]
17c	1.10 ± 0.15[c]	0.79 ± 0.01[c]	1.37 ± 0.06[c]	2.25 ± 0.19[c]
18a	1.66 ± 0.22[c]	2.88 ± 0.19[c]	1.41 ± 0.11[c]	3.39 ± 0.27[c]
18b	1.17 ± 0.14[c]	0.67 ± 0.01[c]	0.90 ± 0.03[c]	1.60 ± 0.08[c]
18c	2.37 ± 0.19[c]	2.25 ± 0.08[c]	2.53 ± 0.47[c]	3.95 ± 0.52[c]

[a]IC_{50}: concentration that inhibits 50% of cell growth. [b]MTT method: drug exposure was for 72 h (means ± SD, n = 3). [c]$^{**}p < 0.001$ vs oridonin group.

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Table 2. IC_{50}[a] Values (μM) of Representative Compounds 16a−c in the Drug-Resistant and Parental Sensitive Cells[b]

compd	SW620	SW620/AD300	NCI-H460	NCI-H460/MX20
oridonin	6.26 ± 0.33	4.67 ± 0.17	17.14 ± 1.06	21.71 ± 0.98
16a	13.95 ± 0.16	14.92 ± 1.28	28.41 ± 0.76	>100
16b	1.96 ± 0.11	1.86 ± 0.06	2.35 ± 0.14	2.91 ± 0.12
16c	2.70 ± 0.07	2.28 ± 0.09	4.81 ± 0.11	7.26 ± 0.28

[a]IC_{50}: concentration that inhibits 50% of cell growth. [b]MTT cytotoxicity assay was assessed in pairs of parental and transporter-overexpressing cell lines: SW620 and ABCB1-overexpressing SW620/AD300 cells; NCI-H460 and ABCG2-overexpressing NCI-H460/MX20 cells.

killed at 5 μM, while only 17.36% normal liver cells were killed. As seen in Table 3, we found that conjugate **16b** has

Table 3. IC_{50}[a] Values (μM) of Compounds 1, 15, 16b, 16c, and Chlorambucil against Human Liver Cancer Cells Bel-7402 and Human Normal Liver Cells L-02

compd	Bel-7402	L-02	SI[b]
oridonin	7.48 ± 0.53	17.78 ± 0.64	2.38
chlorambucil	49.31 ± 1.87	105.78 ± 8.22	2.14
15	>200	>100	
16b	0.50 ± 0.01	4.03 ± 0.36	8.06
16c	1.43 ± 0.09	9.62 ± 0.23	6.73

[a]IC_{50}: concentration that inhibits 50% of cell growth. [b]SI: selective index (IC_{50} on normal cells/IC_{50} on tumor cells).

approximately 8-fold higher selectivity for Bel-7402 cells compared with normal L-02 cells, which was even much higher than that of oridonin. The data suggests that the hybrids from natural product and nitrogen mustards would be safer for the cancer patients receiving chemotherapy.

Drug combinations are widely used for the treatment of cancer to increase the efficiency and reduce side effects.[20] Oridonin has been reported to be combined with anticancer drugs to treat the cancer patients in clinical practice.[21] In order to investigate whether conjugates **16b** and **16c** exhibited their cytotoxicities alone or through the synergistic effect, we tested the potency of equimolar concentration of oridonin, benzoic acid mustard, or **16b** alone, and the combination of equimolar oridonin and benzoic acid mustard on MCF-7 cells. The results showed that the combination of the oridonin and benzoic acid mustard was not superior to conjugate **16b** alone at any concentration. The same phenomenon could also be observed on the compound **16c** (see Supporting Information Figure S1).

As shown in Figure 2, the IC_{50} values of 1:1 combination of oridonin and benzoic acid mustard or chlorambucil was 11-fold or 6-fold higher than that of **16b** or **16c** on MCF-7 cells, respectively. These results indicated that hybrids **16b** and **16c** were more potent than oridonin or an equimolar mixture of its components nitrogen mustard and oridonin. Therefore, the efficacy of **16b** and **16c** in breast cancer could not be mimicked by the simple direct combination.

To determine whether the suppression of cell growth by compound **16b** is caused by a cell-cycle effect, the DNA content of cell nuclei was detected by flow cytometry. As shown in Figure 3, compound **16b** could influence cell cycle progression at low micromolar concentrations. When Bel-7402 cells were treated with different concentrations of compound **16b** (0.125, 0.25, or 0.5 μM), the percentage of cells in G_2 fraction decreased from 19.52% to 4.46% associated with a percentage increase of G_1-phase cells (39.66% to 51.98%, see Supporting Information Table S1).

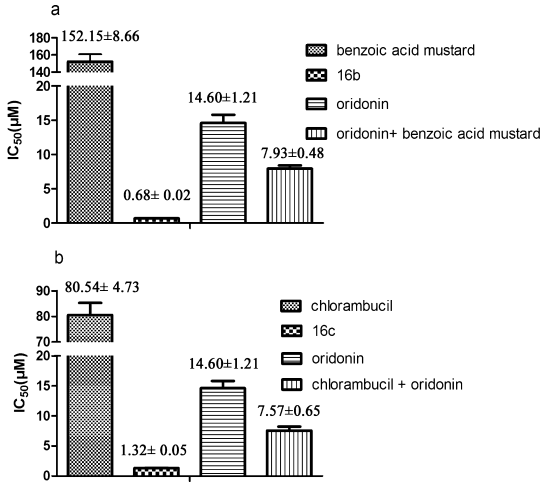

Figure 2. (a) IC_{50} values (μM) of the hybrid (**16b**), oridonin, benzoic acid mustard, and oridonin + benzoic acid mustard against the MCF-7 cells; (b) IC_{50} values (μM) of the hybrid (**16c**), oridonin, chlorambucil, and oridonin + chlorambucil against the MCF-7 cells.

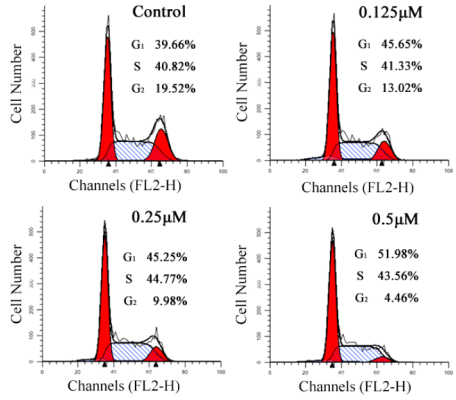

Figure 3. Effects of hybrid **16b** on cell cycle of Bel-7402 cells.

To clarify whether the loss of cancer cell viability promoted by conjugate **16b** is associated with apoptosis, an annexin V-FITC/propidium iodide (PI) binding assay was performed. Bel-7402 cells were treated with vehicle alone or with various concentrations (0.125, 0.25, or 0.5 μM) of compound **16b** for 48 h and then stained with FITC-annexin V and propidium iodide (PI). The percentages of apoptotic Bel-7402 cells were determined by flow cytometry. As shown in Figure 4, conjugate **16b** caused significant induction of apoptosis in a concentration-dependent manner. When treated with 0.125, 0.25, and 0.5 μM compound **16b** for 48 h; the percentages of apoptotic cells were 10.77%, 22.99%, and 48.08% (Q2 + Q4), respectively, compared with 5.21% of vehicle control. This result demonstrated that the antiproliferative activity of hybrid

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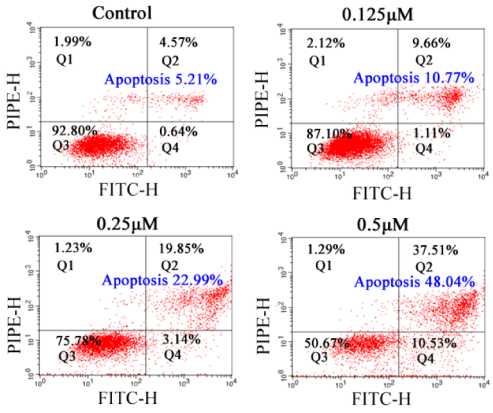

Figure 4. Effects of hybrid **16b** on cell apoptosis.

16b could be attributed to the induction of apoptosis in Bel-7402 cells.

In conclusion, the findings arising from the studies described above open a possible approach to the development of hybrids as potential anticancer agents. In this effort, a series of novel oridonin-coupled nitrogen mustard conjugates were designed and synthesized by following the "combination principle" and their anticancer activities were evaluated against four human cancer cell lines. All the target compounds showed more effective antiproliferative activity than positive control melphalan and chlorambucil. Among them, compound **16b** was the most potent hybrid with IC_{50} values 0.68 ± 0.02 and 0.50 ± 0.01 μM against MCF-7 and Bel-7402 cells, respectively. The promising compounds **16b** and **16c** also exhibited potent antiproliferative activity against drug-resistant cells. Furthermore, it was found that conjugate **16b** has an approximately 8-fold higher selectivity for cancer cells than normal cells, which was higher than those of parent oridonin and clinically used nitrogen mustard drugs. Importantly, it was also found that the antiproliferative activity of the most promising compound **16b** could be attributed to the induction of cell cycle arrest and apoptosis in cancer cells. Collectively, **16b** could be considered as the promising lead compound for the design of more efficacious and less toxic chemotherapeutic agents to enhance the efficacy of chemotherapy in cancer patients.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic methods and characterization of target compounds; procedures for pharmacological activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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