Synthesis and Antitumor Evaluation of Novel Hybrids of Phenylsulfonylfuroxan and Estradiol Derivatives

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Fifteen novel furoxan-based nitric oxide (NO) releasing hybrids of estradiol derivatives were synthesized and evaluated in vitro anti-proliferative activity in MDA-MB-231, A2780, Hela and HUVEC cell lines. Most of them displayed potent anti-proliferative effects. Among the compounds, 4-bromo-3-((phenylsulfonyl)-1,2,5-oxadiazole 2-oxide)-oxy-propoxy-estradiol (11b) exhibited the best activity with IC₅₀ values of 3.58–0.0008 μM. Preliminary pharmacological studies showed that 11b induced apoptosis and hardly affected the cell cycle of MDA-MB-231 cell line. NO-releasing capacity and inhibition of ERK/MAPK pathway signaling might explain the potent antineoplastic activity of these compounds. The preliminary structure-activity relationship (SAR) showed that steroidal scaffolds with a linker in 3-position were favorable moieties to evidently increase the bioactivities of these hybrids. Overall, these results implied that 11b merited to be further investigated as a promising anti-cancer candidate.

1. Introduction

Nitric oxide (NO) that causes vasodilatation was found by Furchgott in the 1980s.[1] NO, as a key signaling molecule synthesized naturally from L-arginine by the action of NO synthase (NOS), plays very important role in a variety of physiological and pathophysiological processes.[2–4] Low levels of NO are able to promote cancer growth as a signal transducer by blood flow regulation, smooth muscle relaxation, neurotransmission, platelet reactivity and so on, while high levels of NO can induce cancer cell apoptosis, inhibit metastasis, and sensitize cancer cells to chemotherapy and immunotherapy.[5–7] Therefore, NO has a double-edged sword effect on the growth of tumors.[6] Furoxan is an important class of NO donors, which can release high levels of NO in vitro.[8] A series of phenylsulfonylfuroxan and anti-tumor drug coupling compounds, such as Furoxan/Doxorubicin hybrid, Furoxan/FTS hybrid and Furoxan/Bifendate hybrid, were reported.[9–11] Compared to their original parent compounds, they have higher anti-tumor activity, safety and drug resistance reversal activity.

One of the most aggressive form of breast cancer,[12] triple-negative breast cancer (TNBC), which usually lacks expression of estrogen receptors (ER), progesterone receptors (PgR), and HER2 protein. Due to consequently unresponsive to both endocrine and anti-HER2 therapy and limiting the therapeutic option, chemotherapy is an alone treatment regimen for these patients.[14–15] Therefore, developing against triple-negative breast cancer drugs is a challenging project and has attracted many researchers to conduct research.

In our previous research, several furoxan-based NO releasing hybrids with the coumarin core or steroidal scaffold were synthesized and exhibited excellent anti-cancer biological activities through inducing apoptosis, disrupting the phosphorylation of MEK1 and ERK1 and inhibition of angiogenesis.[16–18] It is noticeable that the hybrids of phenylsulfonylfuroxan and epioandrosterone/estradiol derivatives show potent anti-proliferative activity in MDA-MB-231 (human triple-negative breast cancer cell line).[18–19] In particular, compounds 1 and 2 (Figure 1) bearing furoxan at 17-position of 3-methoxy estradiol by 2–3 C linkers showed the strong cytotoxicity for MDA-MB-231 cell line with IC₅₀ values of 1.8 and 0.7 nM, respectively.

As we known, the endogenous estrogen metabolite 2-methoxysteradiol (3, 2-ME, Figure 1), formed from estradiol through the action of catechol-O-methyl transferase, has been researched for several decades as a potential anticancer agent resulting from its potent inhibition of tumor neovascularization and cell growth.[20–22] The introduction of methoxy group at the 2-position of estradiol leads to miss estrogen activity, while...
shows a significant anti-cancer action. The result implied that substituent modification on phenyl ring of estradiol might pivotally affect its bioactivity including anti-cancer activity. In consider of our work mentioned above, the interesting anti-cancer activities of hybrids (1 and 2) of furoxan and estradiol using different linkers at 17-position prompted us to further indirectly or directly couple furoxan at 3-hydroxy of 2-ME by with or without linker, and synthesized three 2-ME derivatives with furoxan at its 3-position (5a-c). As control, six hybrids (8a-c, 11a-c) of furoxan and estradiol with 4-bromo or without other group in phenyl ring were prepared by using similar linker type. Moreover, six compounds (14a-c, 16, 18 and 20) including 17-one and 17-alkynyl groups estradiol derivatives merging furoxan also were developed. Herein, we reported design, synthesis and biological evaluation of fifteen novel hybrids of phenylsulfonylfuroxan and estradiol derivatives.

2. Results and Discussion

2.1. Chemistry

As depicted in Scheme 1, in the ethanol solution, 4-bromide estradiol (9) were prepared by the bromination of commercial available estradiol (6) with NBS in 43 % yield. Then, 2-methoxyestradiol (3), estradiol (6), estrone (12) and compound 9 were reacted with 2-bromoethanol or 3-chloropropanol to form intermediates 4a-b, 7a-b, 10a-b and 13a-b in DMF including the present of potassium iodide and sodium hydroxide. Finally, phenylsulfonylfuroxan (21), which was prepared from benzene-thiol according to the reported method in ref. [23], was introduced into compounds 4a-b, 7a-b, 10a-b, 13a-b, 15, 17 and 19 in the presence of 8-diazabicyclo[5.4.0]undec-7-ene (DBU) to synthesize fifteen furoxan and estradiol derivatives coupling compounds 5a-c, 8a-c, 11a-c, 14a-c, 16, 18 and 20 in 10–70 % yields.

2.2. Biological Evaluations

These fifteen newly synthesized hybrids (5a-c, 8a-c, 11a-c, 14a-c, 16, 18 and 20) were evaluated for their cytotoxic effects against MDA-MB-231. As shown in Table 1, all target compounds displayed better anti-proliferative activity against MDA-MB-231 with the IC_{50} values range from 0.00083 to 0.4995 μM than that of control compounds phenylsulfonylfuroxan (21, 1.29 μM IC_{50} value) and 3 (1.27 μM IC_{50} value). Among them, anti-cancer activities with 0.00083–0.3107 μM IC_{50} values of eight compounds 5a-b, 8a-b, 11a-b and 14a-b containing furoxan located to 3-position of estradiol and estrone through 2–3 C linkers were higher than that of furoxan group directly occupied at 3-position four compounds 5c, 8c, 11c and 14c with the IC_{50} values of 0.0384–0.4995 μM. The result was consistent with our previous research mentioned above in compounds 1 and 2. Moreover, 3 C linker four compounds 8a-b and 11a-b had more significant anti-proliferative effects with the IC_{50} values of 0.0083–0.0046 μM against MDA-MB-231. In particular, compound 11b (0.00083 μM) bearing 4-bromo and 3 C linker with furoxan at 3-position was the most potent molecule. It was notable that three hybrids of furoxan and 17β-alkynyl estradiol (16, 18 and 20) also retained the relevant anti-cancer potency possessing the IC_{50} values of 0.0132–0.0553 μM compared to 2-methoxy-3-furoxan-oxy-estradiol 5c (0.0384 μM).

Table 1. Anti-proliferation activities of 5a-c, 8a-c, 11a-c, 14a-c, 16, 18 and 20 in MDA-MB-231.

| Compounds     | MDA-MB-231 (IC_{50} μM) |
|---------------|-------------------------|
| 2-ME(3)       | 1.268                   |
| Furoxan(21)   | 1.293                   |
| 5a            | 0.0183                  |
| 5b            | 0.0212                  |
| 5c            | 0.0384                  |
| 8a            | 0.0045                  |
| 8b            | 0.0022                  |
| 8c            | 0.4995                  |
| 11a           | 0.0046                  |
| 11b           | 0.00083                 |
| 11c           | 0.0780                  |
| 14a           | 0.3107                  |
| 14b           | 0.0190                  |
| 14c           | 0.4228                  |
| 16            | 0.0132                  |
| 18            | 0.0190                  |
| 20            | 0.0553                  |

[a] The data are the mean of triplicate determinations; IC_{50} is the concentration of sample for 50 % cell growth inhibitory rate.
According to the data displayed in Table 1, the preliminary structure-activity relationship (SAR) of the hybrids can be inferred. First, the anti-proliferative activities of the hybrids against MDA-MB-231 were improved with the 2–3 C length of the spacers connecting the NO donors at 3-position of estradiol and estrone. For example, 8b, 11b and 14b with 3 C linker in 3-position showed stronger bioactivities than that of 8a, 11a and 14a containing 2 C linker and without linker compounds 5c, 8c, 11c and 14c, respectively. The information suggested that the linker between steroidal skeleton and furoxan was crucial to preserve the strong anti-cancer activity. Second, 4-bromo substitution of estradiol was more favorable group for anti-proliferation activity compared to 2-methoxy derivative. Thirdly, the replacement of 17-hydroxy in estradiol (5a-c, 8a-c and 11a-c) with 17-carbonyl group (14a-c) slightly decrease anti-proliferation action in MDA-MB-231. Fourthly, the introduction of alkyl group at 17-position of estradiol can keep the strong anti-cancer activity. Overall, the best bioactivity hybrid of furoxan and estradiol with 4-bromo and 3 C linker at 3-position 11b merited to be further investigated as a promised anti-cancer candidate.

Furthermore, including 3 C linker at 3-position of estradiol core three compounds 5b, 8b and 11b were selected to screen their cytotoxic effects against A2780 (human ovary cancer cell lines), Hela (human cervical cancer cell lines) and HUVEC (umbilical vein endothelium cell lines). Interestingly, 5b, 8b and 11b all possessed better bioactivities with the IC_{50} values of 0.104–0.725, 0.053–0.418 and 0.0286–0.056 μM than that of 3 (0.59–1.352 μM) and 21 (1.92–1.475 μM) in A2780 and HUVEC cell lines (Table 2). While in Hela cell lines, activities (3.848 and 3.587 μM) of 8b and 11b were weaker than that of 2-ME and 21 with the IC_{50} values of 0.993 and 3.051 μM, respectively. The results implied that inhibiting action of these compounds have selectivity in different cancer cell lines. Then, their pharmacologic action would be further explored in the next work.

Considering that NO induces cellular apoptosis, our steroidal/furoxan hybrids with the ability to release NO might also have a similar mechanism of inducing apoptosis against tumor cells. To determine the number and stage of apoptotic cells, the annexin-V/PI double staining assay was applied to quantitate 11b treated MDA-MB-231 cell using flow cytometry. As depicted in Figure 2A and 2B, the total proportion of annexin V+/PI− (the right lower quadrant representing early apoptotic) and annexin V+/PI+ (the right upper quadrant representing late apoptotic and necrotic) cells increased from 5.1 to 81.0% after they were exposed to 20, 40 and 80 nM 11b for 24 h.

We next investigated the signaling pathway involved in compound 11b induced apoptosis. Western blot analysis showed that 11b in a dose dependent manner up-regulated the expression and the phosphorylation levels of p53, an important tumor suppressor. Meanwhile, 11b significantly up-regulated the expression of pro-apoptotic protein Bax, and down-regulated the expression of the anti-apoptotic proteins Bcl-2 after treatment with 80 nM 11b. Meanwhile, 11b could also up-regulate the expression and the phosphorylation levels of p53, but might down-regulate the expression of ERK1/2. (D) The profiles showed the proportions (%) in each phase of MDA-MB-231 cells treated with 11b and diluent (DMSO). The experiments were repeated three times.

### Table 2. Anti-proliferation activities of 5b, 8b and 11b in A2780, Hela and HUVEC.

| Compounds | A2780 (IC_{50} μM) | Hela (IC_{50} μM) | HUVEC (IC_{50} μM) |
|-----------|--------------------|-------------------|---------------------|
| 2-ME (3)  | 0.59               | 0.0663            | 1.352               |
| Furoxan (21) | 1.92             | 3.051             | 1.475               |
| 5b        | 0.104              | 2.650             | 0.725               |
| 8b        | 0.053              | 3.848             | 0.418               |
| 11b       | 0.029              | 3.587             | 0.056               |

[a] The data are the mean of triplicate determinations; IC_{50} is the concentration of sample for 50% cell growth inhibitory rate.
In summary, fifteen novel furoxan-estradiol hybrids (5a–c, 8a–c, 11a–c, 14a–c, 16, 18 and 20) were prepared and tested for their in vitro anti-cancer activities. Most of them showed stronger anti-proliferative effects than that of phenylsulfonylfuroxan (25 μM). Furthermore, anti-cancer activity of 11b was diminished by pretreatment with a NO scavenger Hemoglobin in a dose-dependent manner (Figure 3B). The results indicated that the potent anti-proliferation activities may be partly attributed to the release of nitric oxide.

3. Conclusions

In vitro anti-proliferative assay. The in vitro anti-proliferation of the chemical compounds was measured by the MTT reagent. Briefly, as described in the literature,

5 × 10⁴ cells in 100 μL of medium per well were plated in 96-well plates. After being incubated for 24 h, the cells were treated with different concentration of tested compound or DMSO (as negative control) for 48 h. Then the medium with compound or DMSO was replaced with 200 μL of fresh medium containing 10% MTT (5 mg/mL in PBS) in each well and incubated at 37°C for 4 h. The MTT-containing medium was discarded and 150 μL of DMSO per well was added to dissolve the formazan crystals newly formed. Absorbance of each well was determined by a microplate reader (Synergy H4, Bio-Tek) at a 570 nm wavelength. The inhibition rates of proliferation were calculated with the following equation:

\[
\text{Inhibition ratio (\%)} = \left(\frac{\text{OD}_{\text{DMSO}} - \text{OD}_{\text{Comp}}}{\text{OD}_{\text{DMSO}} - \text{OD}_{\text{Blank}}} \right) \times 100
\]

The concentrations of the compounds that inhibited cell growth by 50% (IC₅₀) were calculated using GraphPad Prism, version 6.0. For the NO scavenger experiment, cells were pretreated with the indicated concentrations of hemoglobin (0, 1.25, 2.5, 5, or 10 μM) for 1 h and treated with 100 nM 11b for 24 h. Then the viability of the cells was determined by MTT reagent as described above.

Nitrite measurement in vitro. The levels of NO released by tested compounds in the cells are presented as that of nitrite and were determined by the Griess reagent (Beyotime, China), according to the literature with some modifications. Briefly, cells (1 × 10⁴ per 10 cm dish) were treated with a 100 μM concentration of each compound for 150 min. Subsequently, the cells were harvested and lysed with 100 μL of RIPA lysis buffer (Beyotime, China) for 30 min on ice. The cell lysates were mixed with Griess for 30 min in a dark place, followed by measurement by a microplate reader (Synergy H4, Bio-Tek) at 540 nm wavelength. The cells treated with diluent were used to determine the background levels of nitrite production, while sodium nitrite at different concentrations was measured to generate a standard curve.

Cell apoptosis analysis. Cell apoptosis was detected by flow cytometry according to a previously published method. Briefly, cells were incubated with DMSO or different concentrations of compound 11b for 24 h. The cells were harvested, washed twice with cold 1× PBS, and resuspended in 200 μL of binding buffer at a density of 1 × 10⁵ cells/mL. The cells were then stained with 5 μL of annexin V and PI for 15 min in dark conditions at room temperature and subjected to analysis by flow cytometry (Cytomics FC 500 MPL, Beckman Coulter). The early apoptosis was evaluated based on the percentage of cells with annexin V+ /PI−, while the late apoptosis was that of annexin V− /PI+. The results were indicated as mean values from three independent determinations.

Cell cycle analysis. Cell cycle status was detected by flow cytometry according to a previously published method and were analysed by Multicycle AV (for Windows, version 320) software. Briefly, cells were first treated with DMSO or different concentrations of compound 11b for 24 h and then harvested, washed twice with 1× PBS, and resuspended in 200 μL of 1× PBS. The cells were fixed in 4 mL of ice-cold 75% ethanol at ~20°C overnight and stained with 500 μL of propidium iodide (50 μg/mL, Sigma) containing 0.1% RNase (1 mg/mL, Sigma) for 15 min in dark conditions at room temperature. The cells were then analysed by flow cytometry (Cytomics FC 500 MPL, Beckman Coulter). The results were indicated as mean values from three independent determinations.

Western blot analysis. MDA-MB-231 cells were treated with DMSO or different concentrations of compound 11b for 24 h. Cells were harvested, washed with cold 1× PBS, and lysed with RIPA lysis buffer (Beyotime, China) for 30 min on ice, then centrifuged at 12000 g for 15 min at 4°C. The total protein concentration was determined by BCA protein assay kit (Beyotime, China). Equal amounts (30 μg per load) of protein samples were subjected to
Melting points were measured on a SGW X-4 microsky melting point apparatus without correction. H and 13C NMR spectral data were recorded with a Varian 400 MHz spectrometer at 303 K using TMS as an internal standard. Mass spectra were recorded on Agilent Technologies 1260 infinity LC/MS instrument, and HRMS spectra were recorded on an Agilent Technologies LC/MSD TOF instrument. Analytical and preparative TLC was performed on silica gel HSGF/UV 254. The chromatograms were conducted on silica gel (100–200 mesh) and visualized under UV light at 254 and 365 nm.

3-Phenylsulfonyl-4-hydroxyethylthio-1,2,5-oxadiazole-2-Oxide (21). Synthesis of phenylsulfonylfuroxan from benzenethiol was reported previously in ref. [23].

General procedure for the preparation of 4a–b, 7a–b, 10a–b and 13a–b. To a stirred solution of 3, 6, 9 and 12 (1 mmol) in DMF (5 mL) at room temperature was added corresponding halo alcohol (2 mmol) and K2CO3 (3 mmol). The mixture was refluxed for 2 to 10 hrs and then poured into water (50 mL). After filtration, the residue was washed with water (3 × 10 mL), yielded yellow or white solid 4a–b, 7a–b, 10a–b and 13a–b (40–96%).

General procedure for the preparation of 5a–c, 8a–c, 11a–c and 14a–c 4a–b, 7a–b, 10a–b and 13a–b (1 equiv.) were added to a stirred solution of 21 (1 equiv.) in the presence of 8-diazabicyclo[5.4.0]undec-7-ene (DBU) (3 equiv.) in CH2Cl2 (10 mL). The reaction mixture was stirred at room temperature for 3 hrs and then washed with brine. The organic layer was dried with anhydrous Na2SO4. The solvent was removed under reduced pressure, and the residue was purified by column chromatography (PE:EtOAc = 12:1) to yield 5a–c, 8a–c, 11a–c and 14a–c (10–64%).

2-Methoxy-3-(phenylsulfonyl)-1,2,5-oxadiazole-2-oxide-ethoxy-estriol (5a). Compound 4a (0.20 g) was obtained starting from 3 (1.0 g, 3.3 mmol), yield 17%. Compound 5a (0.15 g) was obtained starting from 4a (0.20 g, 0.58 mmol) and furoxan (0.21 g, 0.58 mmol). 5a: yield 45%; Mp 154–157°C; ESI-MS m/z (%) 593.2 [M + Na]+; 1H NMR (400 MHz, CDCl3) δ 7.50–8.03 (3H, m, –SO2CH3), 6.71–6.84 (2H, m, 1-H, 4-H), 4.40–4.76 (4H, m, –OCH2CH2–), 3.80 (3H, s, 2-OCH3), 3.73 (1H, m, 17-CH), 0.78 (3H, s, 18-CH3). 13C NMR (151 MHz, CDCl3) δ 158.95, 147.92, 145.66, 138.15, 134.55, 134.31, 129.61, 129.25, 128.61, 116.05, 115.00, 110.50, 81.79, 78.03, 76.82, 69.85, 67.16, 56.18, 50.07, 44.33, 43.27, 38.77, 36.78, 30.68, 29.70, 27.31, 26.55, 23.13, 11.10; ESI-HRMS(m/z) [M + Na]+ Calc.: 593.1928, Found: 593.1927.

2-Methoxy-3-(phenylsulfonyl)-1,2,5-oxadiazole-2-oxide-ethoxy-propoxy-estriol (5b). Compound 4b (0.30 g) was obtained starting from 3 (1.20 g, 4 mmol), yield 21%. Compound 5b (0.12 g) was obtained starting from 4b (0.20 g, 0.56 mmol) and furoxan (0.203 g, 0.56 mmol). 5b: yield 37%; Mp 51–53°C; ESI-MS m/z (%) 585.0 [M + H]+; 1H NMR (400 MHz, CDCl3) δ 7.52–8.02 (3H, m, –SO2CH3), 6.66–6.83 (2H, m, 1-H, 4-H), 4.18–4.66 (4H, m, furoxan–OCH3–3-OCH3–), 3.77 (3H, s, 2-OCH3), 3.72 (1H, m, 17-CH), 0.79 (3H, s, 18-CH3). 13C NMR (151 MHz, CDCl3) δ 158.93, 147.60, 146.06, 137.78, 135.52, 132.60, 129.64, 129.04, 128.51, 114.63, 110.49, 109.77, 81.91, 77.03, 76.82, 68.36, 64.96, 56.17, 50.06, 43.39, 38.82, 36.78, 30.67, 29.15, 28.67, 27.35, 26.56, 23.13, 11.10; ESI-HRMS(m/z) [M + H]+ Calc.: 585.2265, Found: 585.2248.
3-((Phenylessulfonyl)-1,2,5-oxadiazole 2-oxide)-oxy-17-estrone

3-((Phenylessulfonyl)-1,2,5-oxadiazole 2-oxide)-oxy-16,17-acetone ketal-17-ethyl-estradiol (18). Compound 18 (0.30 g) was obtained starting from 17 (0.10 g, 0.42 mmol) and furoxan (0.52 g, 1.42 mmol). 18: yield 37%; Mp 77–83°C; ESI-MS m/z (%) 577.2 [M+H]+; 1H NMR (400 MHz, CDCl3) δ 7.63–7.99 (5H, m, –SO2CH2), 6.99–7.35 (3H, m, 1-H, 2-H, 4-H), 4.76 (1H, d, J = 6.9 Hz), 2.58 (1H, s, –CH), 1.47 (6H, s, –(CH2)3). 13C NMR (151 MHz, CDCl3) δ 158.79, 150.41, 138.98, 138.95, 130.08, 135.75, 129.76, 126.85, 126.90, 119.74, 116.90, 112.40, 110.80, 84.75, 83.75, 84.30, 77.02, 76.81, 74.67, 75.90, 46.51, 34.77, 33.91, 30.79, 29.73, 28.67, 25.92, 25.72, 16.93; ESI-HRMS(m/z) [M+H]+ Calc.: 577.2003, Found: 577.1997.

3-((Phenylessulfonyl)-1,2,5-oxadiazole 2-oxide)-oxy-16-hydroxy-17-ethyl-estradiol (20). Compound 20 (0.12 g) was obtained starting from 19 (0.10 g, 0.32 mmol) and furoxan (0.12 g, 0.32 mmol). 20: yield 70%; Mp 120–124°C; ESI-MS m/z (%) 559.0 [M+Na]+; 1H NMR (400 MHz, CDCl3) δ 7.63–8.12 (5H, m, –SO2CH2), 6.99–7.32 (3H, m, 1-H, 2-H, 4-H), 0.93 (3H, s, 18-CH3). 13C NMR (151 MHz, CDCl3) δ 158.81, 150.40, 138.99, 138.08, 137.55, 129.76, 126.66, 126.85, 119.75, 116.91, 110.80, 84.50, 78.35, 78.07, 77.03, 76.82, 73.30, 48.87, 46.63, 43.59, 38.35, 33.95, 29.94, 29.70, 27.58, 25.47, 16.33; ESI-HRMS(m/z) [M+Na]+ Calc.: 559.1509, Found: 559.1503.

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Conflict of Interest

The authors declare no conflict of interest.

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