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SAR studies of 2-methoxyestradiol and development of its analogs as probes of anti-tumor mechanisms

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Abstract—The major estrogen metabolite 2-methoxyestradiol (2ME) has been shown to target tumor cells without severe side effects and is currently being evaluated in clinical trials for several types of cancer. Despite its promise for use in clinical setting, the mechanism(s) by which 2ME exerts its anti-tumor activity is not clearly defined at this time. Employing organic chemistry tools, we synthesized 2ME analogs with which 2ME affinity column was prepared, enabling us to detect a protein that selectively interacts with 2ME. This 2ME analog will be useful as a probe to identify the biological target(s) of 2ME and study their functions in tumor cells.

Numerous bioactive natural products that exert their activities via inhibition of specific intracellular targets have been isolated from various sources over the years. Several natural products have been directly employed as a lead compound in drug development efforts. Recently, these natural products are increasingly serving an additional purpose in the exploration of cell biology through mechanistic action studies, an approach called 'chemical genetics.'[1–4] In this approach, the identification of a natural product's target protein serves as the starting point for exploring complex signaling pathways associated with pathological disorders that can be inhibited by the natural product.

Estrogens (Fig. 1) have been shown to promote tumor growth, mainly in breast cancer models. Furthermore, many estrogen-like molecules from various sources have drawn concerns as potential carcinogens. The natural product 2-methoxyestradiol (2ME) is a major estrogen metabolite found in human blood and urine, the product of the sequential hydroxylation and methylation of estradiol by enzymes cytochrome P450 and catechol-*O*-methyltransferase (COMT) mainly in the liver and erythrocytes. In contrast to most estrogens that have long been suspected as cancer-promoting agents, 2ME has been shown to inhibit tumor growth, as a result has been referred to as 'a good estrogen'.[5]

Unlike the majority of anti-tumor agents, 2ME has been shown to target both tumor cells and their blood supply.[6] In addition, 2ME has been shown to possess potent chemopreventive [6–9] and radio-sensitizing effects.[10,11]

There have been extensive efforts to understand the molecular basis for the mechanism of action of 2ME. Although several signaling pathways inhibited by 2ME have been suggested,[12–14] the molecular mechanism(s) by which 2ME exerts these activities is not clearly defined at this time. Despite structural similarities between 2ME and other cancer-promoting estrogens that act through estrogen receptor (ER), it has been demonstrated that the action of 2ME is not mediated via ER.[15–20] To better understand the modes of 2ME action, identification of its cellular target(s) is an important first step. Given that 2ME is currently being evaluated for clinical use for several types of cancer, its cellular target(s) will be of considerable clinical relevance in cancer therapy and may be used to develop therapeutic agents with better efficacy. Moreover, the modes of 2ME action study may reveal novel signaling pathways.

In this study, we synthesized 2ME analogs to investigate SAR of 2ME and prepared a 2ME affinity column with which 2ME binding protein(s) were detected. This

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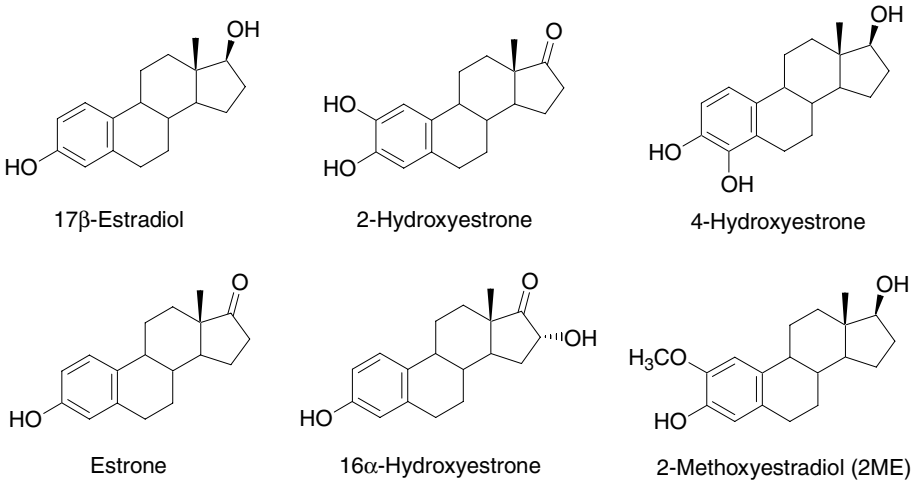
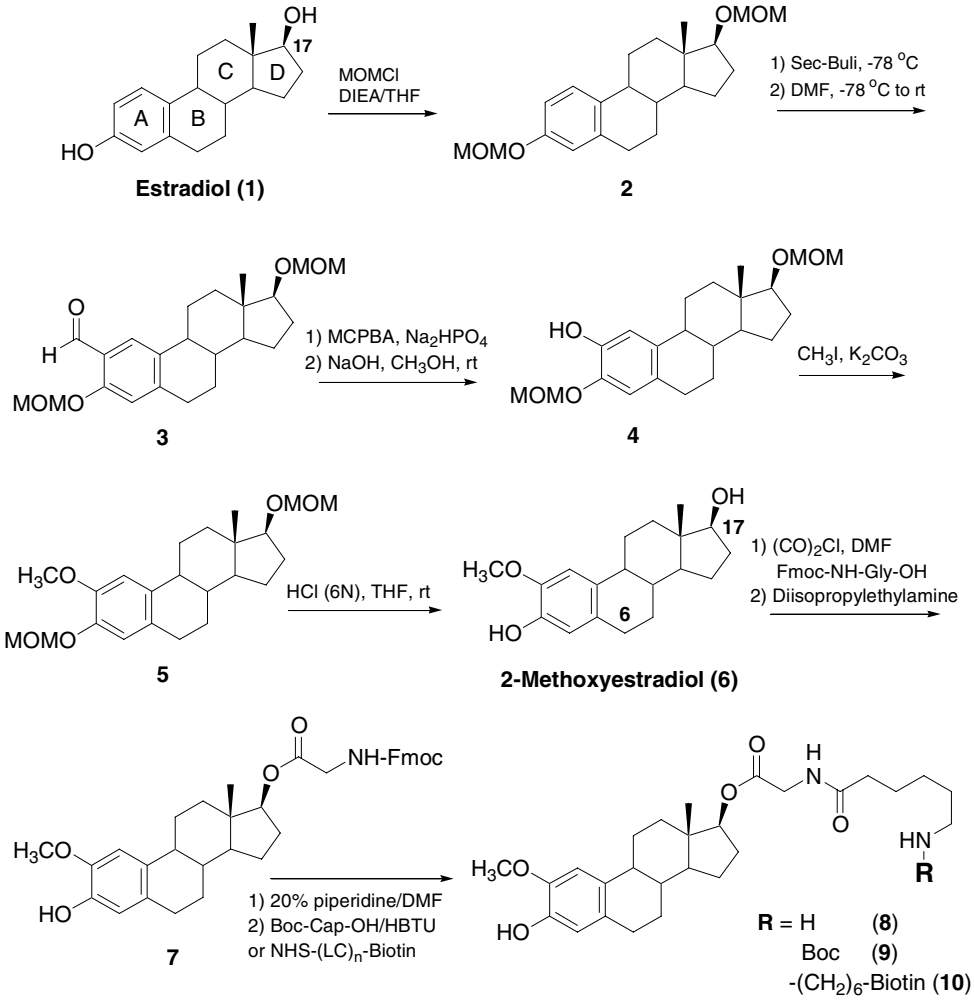


Figure 1. Estrogen metabolites derived from estradiol.

affinity chromatographic approach has been successfully utilized for a number of natural products.[21–24] In this approach, the key step is the addition of a molecular handle to 2ME that does not destroy its activity after attachment to an affinity column.

Given its important biological activities, there has been considerable interest in synthesizing 2-ME and its synthetic derivatives, yielding several efficient synthetic approaches as well as information on preliminary structure-activity relationship (SAR).[25–28] For example,



Scheme 1. Synthetic strategy of 2ME O-17 derivatives.

modifications of the A- or B-ring resulted in significant decrease in the anti-proliferative activity, suggesting that the A- and B-ring may be critical for the activity of 2ME. In contrast, modifications at the D-ring resulted in rather intriguing effects on the anti-tumor activity of 2ME. For example, an isosteric replacement of hydroxyl group at the C-17 with fluorine completely abolished the anti-tumor activity of 2ME. However, when the C-17 hydroxyl group is replaced with a methylene group, its biological activity was restored to the level of 2ME.[29] Based on this SAR information, a molecular handle used to generate a 2ME affinity resin can likely be attached to the D ring of 2ME without a loss of the anti-tumor activity.

With this in mind, a synthetic strategy shown in Scheme 1 was developed, and a number of 2ME O-17 derivatives was synthesized (Fig. 2).[30] To determine whether these derivatives retain the anti-proliferative activity of 2ME, the anti-proliferative activity of derivatives were measured in LNCaP prostate cancer cells (Table 1).

The biological activities of 2ME O-17 derivatives are quite interesting. For example, while 2ME-Gly-NH_2 (**11**) is still partially active, 2ME-Gly-Fmoc (**7**) shows no activity against LNCaP prostate cancer cells (Table 1). It is likely that the bulky Fmoc group in close proximity to 2ME pharmacophore may block the free access of the pharmacophore to a defined 2ME binding pocket of target protein due to steric hindrance, resulting in the loss of biological activity. Meanwhile, compounds 2ME-Gly-Cap-Boc (**9**) and 2ME-Gly-Cap-Biotin (**10**), which have a much longer linear linker at the O-17 position compared to compound 2ME-Gly-Fmoc (**7**), showed an anti-proliferative activity comparable to 2ME, despite the presence of bulky *t*-butyloxy carbonyl (Boc) group at the end of the linear linker.

Based on these observations, a long linear linker of the type used in compounds **8**–**10** may play a key role in retaining the activity of 2ME. This result indicates that 2ME binding target(s) may possess a well-defined 2ME binding pocket.

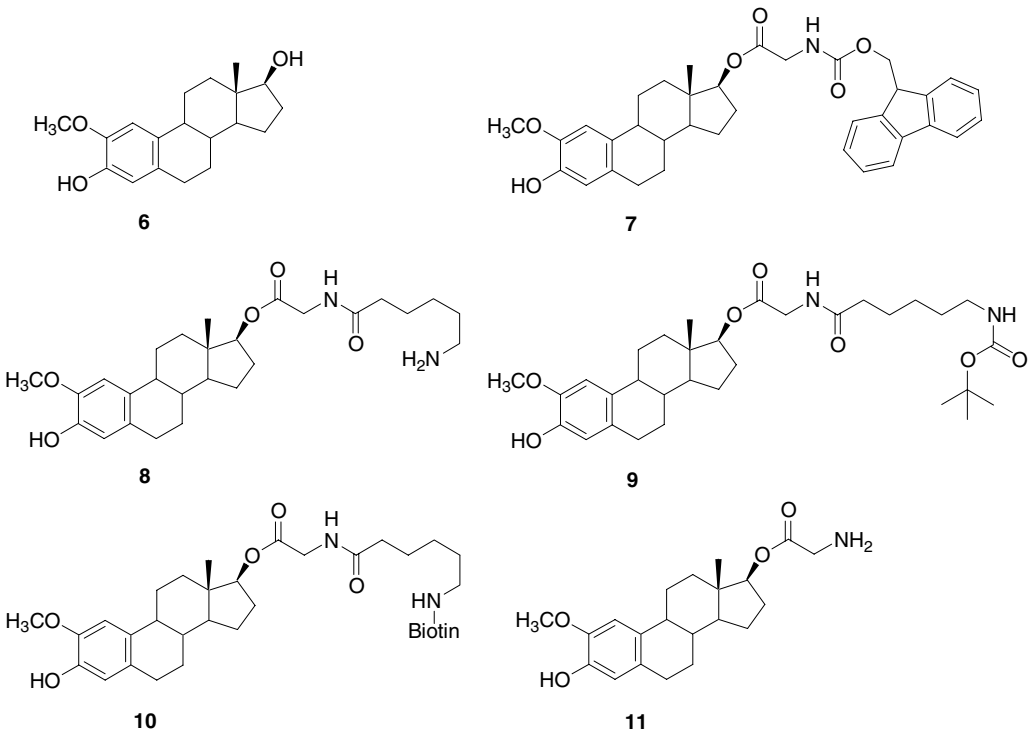


Figure 2. 2ME O-17 derivatives. The activity of 2ME analogs was evaluated by analysis of inhibition of cell proliferation against LNCaP prostate cancer cells.

Table 1. Anti-proliferative activity of O-17-substituted 2-methoxyestradiol (2ME) on LNCaP human prostate cancer cells

Compound	Compound name	IC_{50}[a] (μM)
6	2ME	0.5
7	2ME-Gly-Fmoc	No inhibition (50 <)
8	2ME-Gly-Cap-NH_2	0.6
9	2ME-Gly-Cap-Boc	0.6
10	2ME-Gly-Cap-Biotin	0.6
11	2ME-Gly-NH_2	0.9

[a] Values are means of three experiments. The cell number was counted after 24 h incubation at 37 °C.

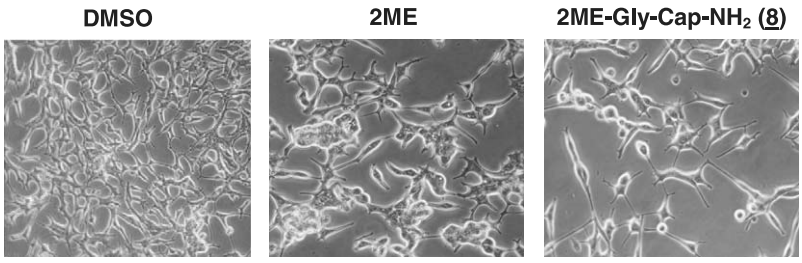


Figure 3. LNCaP cells treated with DMSO, 2ME (5 μM) or compound **8** (5 μM) for 48 h. Photographs were taken using a Kodak digital camera.

Next, we wanted to test whether compound **8** (2ME-Gly-Cap-NH_2) has the same effect as that of 2ME in tumor cells. LNCaP human prostate cancer cells were treated with vehicle (DMSO), 2ME (positive control) or compound **8** at 37 °C. After 48 h incubation, cells were observed using a light microscope. As shown in Figure 3, 2ME and compound **8** caused a similar morphology change and apoptosis, suggesting that compound **8** retains the activity of 2ME, despite an extra molecular handle.

With the information on 2ME O-17 derivatives, a 2ME affinity matrix was prepared from a coupling reaction of compound **8** with Affigel-10 (Bio-Rad). Affigel-10 has been widely used for the affinity column chromatographic purposes over the years.[31,32] Using routine solid-phase chemistry, we prepared an 2ME-Affigel matrix from the reaction of a free amine group of compound **8** with *N*-succimidyl group of Affigel-10 in DMF solvent (Fig. 4).[33]

Next, the ability of the affinity resin to bind any 2ME binding protein from cell extracts derived from cancer cells was examined. Exponentially growing LNCaP cancer cells were lysed with lysis buffer. The lysates were then incubated with the 2ME-linked Affigel-10 beads with or without a 1-fold or 5-fold excess of 2ME at 4 °C overnight. After unbound proteins were washed with buffer, the Affigel-bound proteins were collected by boiling the beads. It should be noted that the interaction between 2ME binding protein(s) and 2ME is likely to be non-covalent. After gel-electrophoresis, the affinity bead-bound proteins were silver-stained (Fig. 5).

As shown in Figure 5, a protein band (2ME binding protein, MBP) was efficiently competed away by 5-fold excess 2ME, indicating a specific interaction between MBP and 2ME. When cell lysates were incubated with inactive 2ME analog **7**, a 92-kD protein band, but not MBP, was competed away (see Supplementary information). These data show for the first time that the anti-tumor estrogen metabolite 2ME may selectively bind a protein other than microtubules.

In conclusion, we have established SAR at the O-17 position of 2ME and generated a number of O-17 derivatives that retain anti-tumor activity of 2ME. This allowed us to prepare 2ME affinity matrix and detect

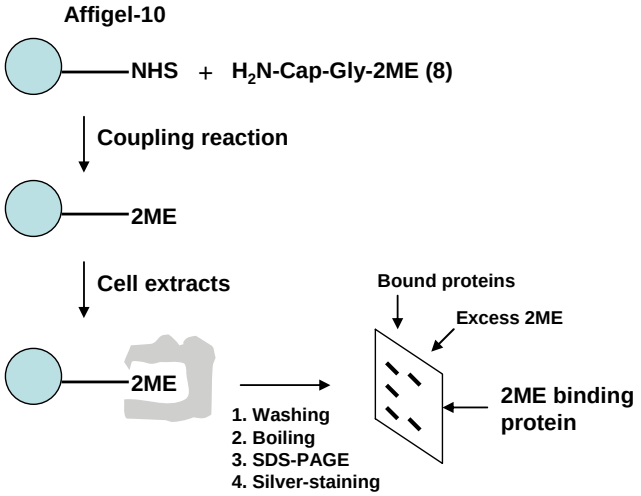


Figure 4. Affinity chromatographic approach using Affigel-10.

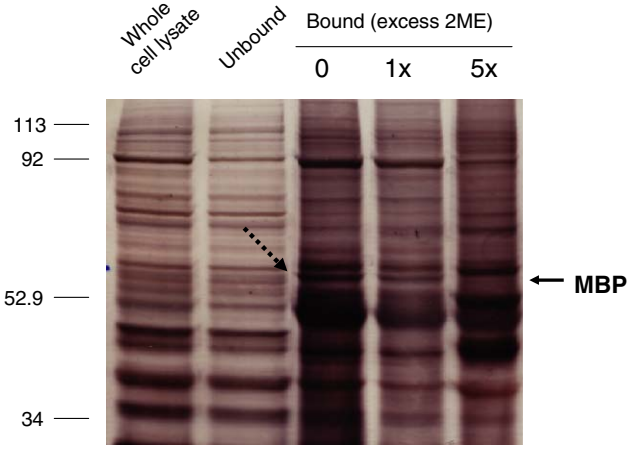


Figure 5. Cancer cell extracts were incubated with 2ME-Affigel-10 affinity matrix with and without a 1-fold or 5-fold excess of 2ME, and the bound-proteins were resolved by SDS–PAGE and the gel was silver-stained to visualize the affinity matrix-bound proteins. To ensure equal loading of protein samples, BioRad Bradford-based protein assay was carried out before SDS–PAGE.

binding protein that specifically interacts with 2ME, thus allowing the identification of the protein target using protein purification techniques and mass-spec. studies. The identification of the 2ME binding protein may prove useful in the study of the modes of 2ME action.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.04.030.

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30. (a) The synthesis of 2ME is described previously[30a]; (b) The synthesis of 2ME-Gly-Cap-Biotin (**10**) is described as the representative synthetic procedure for 2ME O-17 derivatives: Fmoc-Gly-OH was coupled to 2ME at O-17 to give **7**. Specifically, dimethylformamide (DMF, 30μL) was added to methylene chloride (CH_2Cl_2, 10 mL) containing Fmoc-Gly-OH (6.7 mmol) and oxalyl chloride (17.2 mmol). After 3 h of stirring, solvent was removed under nitrogen and re-dissolved in CH_2Cl_2(10 mL). The solution was combined with 2ME (2.2 mmol) and dimethylaminopyridine (DMAP, 18 mmol) in CH_2Cl_2 at 0 °C. After 3 h of stirring at room temperature, the concentrated reaction mixture was subjected to flash column chromatography to yield **7** (1.2 mmol, 54%). Compound **7** was then treated with 20% piperidine in DMF to yield **11**. EZ-Link NHS-LC-Biotin (Pierce, 36 μmol) was dissolved in DMF (0.5 mL) containing **11** (28 μmol). After stirring overnight at rt, solvent was removed and subjected to flash column chromatography to yield the final product **10** (20μmol, 72%). The final product was characterized by ^{1}H NMR (Varian, 400 MHz) and mass spectroscopy. ^{1}H NMR: $\delta = 6.78$ (s, 1H, 1-H), 6.62 (s, 1 H, 4-H), 4.73 (t, $^3J = 8.4$ Hz, 1H, 17-H), 4.52 (m, 1H, 6-H_{Bio}), 4.42 (m, 1H, 3-H_{Bio}), 3.85 (s, 3H, 2-OCH_3), 3.24 (m, 1H, 4-H_{Bio}), 3.18 (m, 2H, 1′-H_{Bio}), 2.92 (m, 1H, 6-Hx_{Bio}), 2.74 (d, $^2J = 12.8$ Hz, 1H, 6-Hy_{Bio}) 2.71 (s, 2H, 2-H_{Gly}), 0.84 (s, 3H, 13-CH_3). MS (MALDI): $m/z = 669$, calcd for $C_{37}H_{54}N_4O_7S{\cdot}Na$: $m/z = 668.89$.
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33. (a) Preparation of 2ME affinity resin: Affigel-10 (Bio-Rad) washed with DMSO was incubated with 2ME-Gly-Cap-NH_2 (**8**) overnight at 4 °C on rocking shaker. Affigel-10 was washed with DMSO, 1 N acetic acid, and distilled water sequentially and stored in 0.05% sodium azide in distilled water before needed.; (b) Purification of 2ME binding protein (MBP): purification. Cytosolic and nucleic lysates were precleaned with protein A/G agarose (Santa Cruz Biotechnology). After centrifugation, lysates containing equal amounts of total protein were incubated with 2ME-linked Affigel-10 with and without a 1-fold and 5-fold excess of 2ME on rocking shaker for 30 min at 4 °C. Unbounded proteins were extensively washed with 50 mM Tris and bound proteins were eluted. After SDS–PAGE, the 2ME binding proteins were silver-stained and dried.