Bioorganic & Medicinal Chemistry Letters 19 (2009) 6241–6244

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, antiproliferative, and pharmacokinetic properties of 3- and 17-double-modified analogs of 2-methoxyestradiol

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ARTICLE INFO

Article history:
Received 25 June 2009
Revised 4 August 2009
Accepted 5 August 2009
Available online 8 August 2009

Keywords:
Antitumor
Antiproliferative
2-Methoxyestradiol analogs

ABSTRACT

The syntheses of 21 analogs of 2-methoxyestradiol are presented, including ENMD-1198 which was selected for advancement into Phase 1 clinical trials in oncology. These analogs were evaluated for antiproliferative activity using breast tumor MDA-MB-231 cells, for antiangiogenic activity in HUVEC proliferation assays, and for estrogenic activity in MCF-7 cell proliferation. The most active analogs were evaluated for iv and oral pharmacokinetic properties via cassette dosing in rat and in mice pharmacokinetic models.

2-Methoxyestradiol (2ME2, Panzem®) was originally identified as an endogenous metabolite of estradiol, and has antiproliferative and antiangiogenic activity when dosed at pharmacological levels in in vitro and in vivo oncology models.[1] The primary mechanisms of action for 2ME2 are thought to be inhibition of tubulin polymerization by binding to the colchicine binding site on tubulin and inhibition of HIF-1α.[2] Phase 1 and Phase 2 oncology clinical trials of 2ME2 showed that it, like other estranes, undergoes extensive metabolism involving conjugation (glucuronidation, sulfation) at positions 3 and 17 and oxidation to 2-methoxyestrone.[3]

Most prior structure–activity relationship (SAR) studies for 2ME2 analogs focused on making single point modifications to the A, B or D rings of the steroid to increase inhibition of tubulin polymerization or antiproliferative activity.[4] Our data indicated that the principal liability of 2ME2 was its rapid metabolism, rather than insufficient activity *per se*. This suggested that analogs designed to reduce metabolism could be better drug candidates than analogs selected solely on the basis of in vitro tubulin or antiproliferative activity. Additionally, our previous SAR studies indicated that single modifications at the 3, 16, or 17 positions were not sufficient to provide the desired improvement in metabolic profile and/or pharmacological activity.[4–6]

In this Letter, we report simultaneous modification of positions 3- and 17- of 2ME2 using substituents that increase metabolic stability, and increase or maintain in vitro potency. The substituents selected were based on our previous work from a series of analogs modified individually at the 3 or 17 positions of 2ME2.[5,6] The novel double-modified analogs were evaluated in vitro for inhibition of tumor cell and angiogenic proliferation, and for estrogenicity. Analogs with acceptable in vitro profiles were assessed for pharmacokinetic (PK) properties by cassette dosing in rats or in CD1 mice. These studies ultimately lead to selection of several lead candidates for pharmacological evaluation, including ENMD-1198, which is currently in Phase 1 oncology clinical trials (Fig. 1).[7]

The synthetic strategy used in this study is shown in Schemes 1 and 2. 2-Methoxyestrone (**1**)[8] was modified at position 17 (Scheme 1) by Wittig coupling to give **2** (**2 a** R^1 = H 75% yield, **2b** R^1 = Me 84% yield).[9] The methylene olefin can be reduced to the 17-β-methyl isomer (**3**, quantitative) by catalytic hydrogenation.[10] Alternatively, **1** can be reduced to 17-deoxy analog (**4**, 90%) by a Wolf–Kishner reduction.[11] The 16,17-olefin analog **5** can be prepared by Shapiro reduction of **1** (90%).[12] Further modification of **2**, **3**, **4** or **5** at position 3 as depicted in Scheme 2 gave the double-modified analogs used in this study.

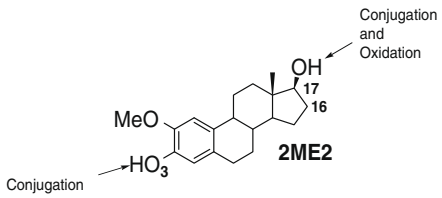

Figure 1. Structure of 2-methoxyestradiol showing oxidation and conjugation sites.

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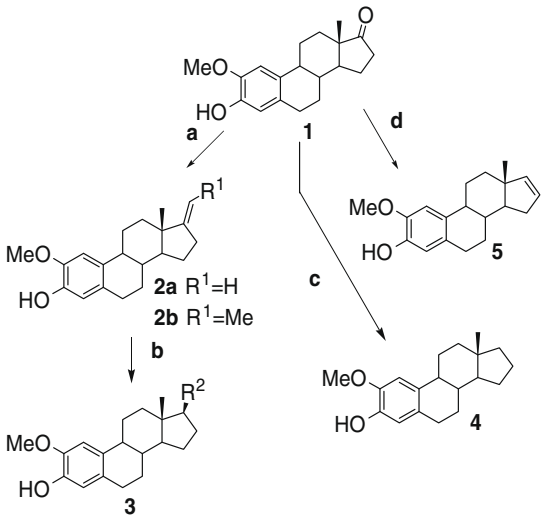
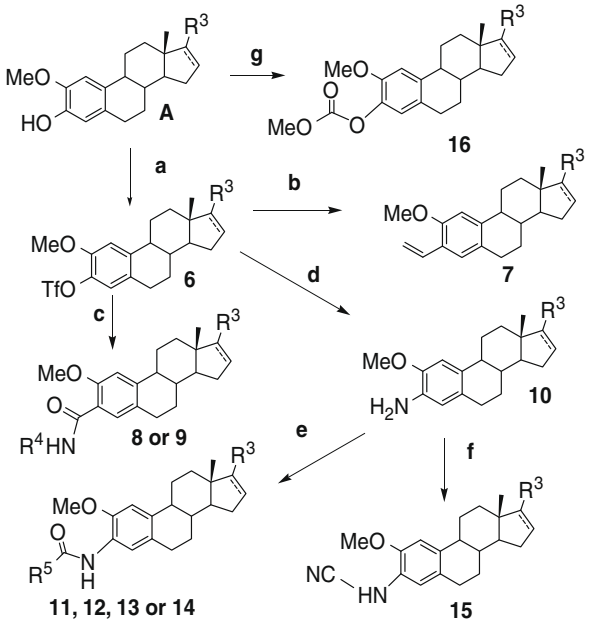
doi:10.1016/j.bmcl.2009.08.020



Scheme 1. Modification of 17-position of 2ME2. (a) BuLi, $(Ph)_3PR$, toluene (R^1 = H(**2a**) or Me(**2b**)); (b) H_2, Pd/C EtOAc ($R^2 = CH_3$(3)); (c) NH_2NH_2, NaOH; (d) (1) *p*-tosyl sulfonamide, (2) BuLi.



Scheme 2. Modification of 3-position of 17-substituted-2-methoxyestranes. R^3 substituents are shown in Table 1. (a) Tf_2O, pyridine, 0 °C to rt; (b) $(Bu)_3SnCHCH_2$, $Pd(Cl)_2PPh_3$, 2,6-di-*tert*-butyl methyl phenol, LiCl; (c) $Pd(OAc)_2$, dppf, CO, KOAc, DMSO, 55 °C then $SOCl_2$ at reflux, then $EtNH_2$, THF R^4 = Et (**8**) or $Pd(Cl)_2$, dppp, CO, HMDS, DMF, 100 °C, MeOH, then acidic workup R^4 = H (**9**); (d) $Pd(OAc)_2$ *rac*-BINAP. Cs_2CO_3, benzophenone imine, toluene, reflux, then 2 M HCl, THF; (e) formic acid, *N,N*-carbonyldiimidazole, THF (R^5 = H, **11**) or Ac_2O, 10 M NaOH 0 °C to rt ($R^5 = CH_3$, **12**) or NaOCN, H_2O, AcOH ($R^5 = NH_2$, **13**) or methyl chloroformate, Et_3N, DCM ($R^5 = OCH_3$, **14**); (f) BrCN, Et_2O, DCM 0 °C to rt; (g) methyl chloroformate, DMAP, DCM.

3-Position modifications are shown in Scheme 2. Initially, for analogs of general formula **A** (**1**, **2a**, **2b**, **3**, **4** or **5** from Scheme 1) the 3-hydroxy group was converted to the 3-triflate (**6**, 90%) using pyridine and triflic anhydride. The resulting triflate can undergo Pd-catalyzed vinylation using vinyl tributyl tin to give **7** (30%).[13] Similarly, **6** can undergo Pd-mediated CO insertion to give a carboxylic acid[14] which can be converted to a secondary amide (**8**, 50**%**, two steps). Alternatively, the primary amide was generated directly from **6** via a CO insertion reaction using HMDS as the nitrogen source to give **9** (50–75%).[15] Triflate **6** was converted to amine **10** by Pd-mediated benzophenone imine formation and subsequent acid hydrolysis (70–77%, two steps).[16] Amine **10** was converted to the formamide (**11**, 78–88%) by in situ preparation of formyl imidazolidine from formic acid and carbonyl diimidazole in THF followed by amide coupling. Acetamide (**12**, 85**%**) was prepared from acetic anhydride and **10** under basic conditions. Urea **13** was prepared from amine **10** using sodium cyanate under acidic conditions (50%).[17] Carbamate **14** was prepared from **10** using methyl chloroformate with triethylamine (60%).[18] Aminonitrile **15** (40%) was prepared using cyanogen bromide from **10**.[19] Carbonate **16** was prepared directly from **A** using methyl chloroformate with DMAP (60%).

The in vitro antitumor and antiangiogenic activities are summarized in Table 1. All compounds were screened in HUVEC proliferation assays[7] as a surrogate for antiangiogenic activity, on MDA-MB-231 tumor cells[7] for antitumor activity, and for ability to sustain proliferation of estrogen-dependant MCF-7 cells (estrogenicity). Desirable characteristics of the analogs were maintained or improved antiproliferative activities (decreased IC_{50} values) and decreased estrogenicity (stimulation index <1.0 at any concentration compared to 2ME2).

The 3- and 17-moieties used to design this series of doubly modified 2ME2 analogs were chosen based on in vitro activity and/or PK characteristics as individual substituents from our prior studies. [5,6] In general, all activities presented in the discussion were compared to 2ME2 as a benchmark.

Doubly modified 3-amines **10a**–**10e** showed a moderate to significant decrease in in vitro activity. Amines **10b** and **10c** had a slight decrease in antiproliferative activity but were equivalent in HUVEC proliferative activity. Amines **10a**, **10d** and **10e** had significantly less activity in both antiproliferative and antiangiogenic activities. 3-Aminonitrile **15** was slightly more active in in vitro proliferation assays.

In the carboxamide series **9**, the order of potency for the 17 substituent in MDA-MB-231 antiproliferative activity was: 17-methylene (**9b**) > 16,17-olefin (**9d**) > 17-deoxy (**9c**) > 17-oxo

Table 1
In vitro activities of double-modified analogs of 2ME2

#	3-Subst.	17-Subst.	MDA-MB-231 IC_{50} (μM)	HUVEC IC_{50} (μM)
2ME2	–OH	–OH	0.79 ± 0.14	0.68 ± 0.15
7	–CH=CH_2	–H	1.86 ± 0.22	0.58 ± 0.04
8	–CONH(Et)	–H	53.01 ± 9.67	37.98 ± 2.37
9a	–$CONH_2$	=O	2.20 ± 0.43	5.46 ± 1.57
9b	–$CONH_2$	=CH_2	0.12 ± 0.06	0.25 ± 0.01
9c	–$CONH_2$	–H	1.86 ± 0.86	1.19 ± 0.33
9d	–$CONH_2$	–H[a]	0.20 ± 0.1	0.13 ± 0.05
10a	–NH_2	=O	46.91 ± 8.15	23.42 ± 4.14
10b	–NH_2	=CH_2	2.11 ± 0.57	1.00 ± 0.19
10c	–NH_2	=$CHCH_3$	1.94 ± 0.27	0.72 ± 0.10
10d	–NH_2	–H	7.38 ± 1.66	4.72 ± 0.49
10e	–NH_2	–Me	7.31 ± 0.58	2.65 ± 0.77
11a	–NHCOH	=O	1.22 ± 0.60	0.53 ± 0.22
11b	–NHCOH	=CH_2	0.30 ± 0.08	0.22 ± 0.03
11c	–NHCOH	=$CHCH_3$	0.56 ± 0.02	0.58 ± 0.00
11d	–NHCOH	–H	8.29 ± 1.64	5.80 ± 0.73
11e	–NHCOH	–Me	0.61 ± 0.00	0.59 ± 0.00
12	–NHCOMe	–H	9.25 ± 0.66	1.68 ± 0.32
13	–$NHCONH_2$	=O	0.71[b]	0.61[b]
14	–NHCOOMe	=O	5.60 ± 1.42	8.22 ± 2.79
15	–NHCN	=O	0.20 ± 0.12	0.25 ± 0.02
16	–OCOOMe	=O	24.99[b]	32.87 ± 11.43

[a] 16, 17 Olefin.
[b] Standard deviation not available where results are reported from a single experiment.

(**9a**) and ranged from sixfold more to threefold less potent than 2ME2. For HUVEC proliferation assay the rank order was: 17-methylene (**9b**) > 17-deoxy (**9c**) > 16,17-olefin (**9d**) > 17-oxo (**9a**) and were fivefold more to eightfold less potent compared to 2ME2. In this series, the 17-methylene and 16,17-olefin had greatest in vitro potency and the deoxy showed a modest drop in activity.

In the formamide series, the order of antitumor potency was: 17-methylene (**11b**) > 17-ethylene (**11c**) ~ 17-methyl (**11e**) > 17-oxo (**11a**) > 17-deoxy (**11d**) and ranged from twofold more to 10-fold less potent in tumor cell antiproliferative activity. HUVEC antiproliferative activity was ranked: 17-methylene (**11b**) > 17-oxo (**11a**) > 17-ethylene (**11c**) ~ 17-methyl (**11e**) > 17-deoxy (**11d**) and were threefold more to eightfold less potent compared to 2ME2. Substituents with sp^2 hybridization such as the methylene, ethylene and oxo were the most active analogs in this series. Addition of steric bulk to the exocyclic olefin brought a modest drop in activity (compare **11b** to **11c**). In this series, the deoxy substitution had the greatest dropoff in activity.

Since some of the most potent analogs (**9** and **11**) in this series contained carbonyl substituents at position 3, we chose to investigate the SAR of related substituents on the doubly substituted scaffold. From this investigation, we found that as steric bulk increased, antiproliferative activity decreased. For example, ethyl carboxamide (**8**), 3-acetamide (**12**), 3-methylcarbamate (**14**), and 3-methyl carbonate (**16**) all showed decreases in MDA-MB-231 and HUVEC antiproliferative activity compared to **9** and **11**. Conversely, when steric bulk did not increase at position 3, we maintained in vitro potency as illustrated by the 3-vinyl (**7**) and 3-urea (**13**).

It was desirable to maintain the significantly reduced estrogenicity of 2ME2 compared to estradiol. The substituents evaluated in this study had been selected partially on a basis of low degree of estrogenicity as single substituents.[5,6] All analogs with significant in vitro activities were evaluated using the estrogen-dependent MCF-7 in vitro proliferation assay as a surrogate for estrogenicity.[7] As expected from our reported data on individual substitutions,[5,6] all double-modified analogs tested had equivalent or less estrogenic activity than 2ME2 (data not shown).

Rat cassette dosing was used as an initial screen for PK evaluation in our discovery program. An advantage of this technique is that several analogs can be evaluated simultaneously in a single animal.[20] Compounds **9a**, **9b**, **9c**, **9d**, **10b**, **10c**, **11b**, **11c**, **11d**, **11e**, and **15** were evaluated and results are presented in Table 2. Aminonitrile **15** had no oral bioavailability. Amines **10c** and **10b** had the greatest oral C_{max}. Formamide **11d** and carboxamide **9d** had approximately equal oral (PO) C_{max} PO. Most analogs had similar PO AUC with the exceptions being carboxamide **9a**, amines **10b** and **10c** and formamide **11b**. All analogs had significantly higher oral bioavailability (%*F*) compared to 2ME2.

Based on these data, formamides (**11b**,**c**,**d**,**e**), amines (**10b**,**c**) and carboxamides (**9a**,**b**,**c**,**d**) had acceptable PK properties in cassette dosing analysis. However, the amines were not as active in vitro as the best formamides or carboxamides. Furthermore, primary aromatic amine toxicity is well known.[21] The lead formamides have a risk of aniline-like toxicity profile, since they could act as prodrugs for anilines via cleavage of the formamide. Carboxamides will not generate the same metabolite and were considered to have lower risks of toxicity. This, along with their in vitro profiles, resulted in several of the carboxamides being selected for evaluation in mouse single-compound PK in preparation for mouse tumor models.

Carboxamides **9b**, **9c** and **9d** were tested by oral administration in CD1 mice and compared to 2ME2. Data are presented in Table 2. All three double-modified analogs had much higher AUC and longer $t_{1/2}$ than 2ME2. Compound **9d** has the highest measured C_{max} and AUC of the analogs tested—fivefold and 23-fold higher than 2ME2, respectively. Compound **9b** had the longest $t_{1/2}$ for this set of analogs (4.8 h), while **9d** and **9c** had a $t_{1/2}$ of 2.1 h and 1.1 h, respectively, compared to 0.3 h for 2ME2. Based on favorable PK and in vitro properties compared to 2ME2, carboxamides **9b**, **9c** and **9d** were selected as leads for further development. The results of these studies have been communicated elsewhere.[7]

Table 2
In vivo rat and mouse PK summary

#	Rat cassette dosing[a]			Mouse PK[a,b]		
	PO C_{max} (nM)	PO AUC (nM h)	*F* (%)	PO C_{max} (nM)	PO AU $C_{0-\infty}$ (nM h)	$t_{1/2}$ (h)
2ME2	BLOQ[c]	BLOQ[c]	—	294	142	0.3
9a	47	61.5	13.6	nd	nd	nd
9b	62	467	22.8	334	3196	4.8
9c	130	624	14.9	504	2527	1.1
9d	42	320	14.3	1427	3359	2.1
10b	150	1273	19.3	nd	nd	nd
10c	190	2146	27	nd	nd	nd
11b	127	1703	39.5	nd	nd	nd
11c	83	659	24.7	nd	nd	nd
11d	53	508.2	24.7	nd	nd	nd
11e	73	673.2	20.6	nd	nd	nd
15	BLOQ[c]	BLOQ[c]	—	nd	nd	nd

[a] Pharmacokinetic variables for compounds were determined using liquid ion chromatography–tandem mass spectrometric analysis. Pharmacokinetic data were modeled using noncompartmental analysis. Oral bioavailability (%*F*) was calculated from the ratio of oral (PO) and intravenous area under the curve (AUC) values.
[b] All compounds were dosed at 45 mg/m^2.
[c] At the low dose used for cassette dosing, values for these analogs were below the limit of quantization.

In conclusion, twenty-one 3- and 17-double-modified analogs of 2ME2 were synthesized and evaluated for antiproliferative activity using MBA-MB-231 cells, and antiangiogenic activity using HUVEC proliferation. The most potent carboxamide analogs **9b** and **9d** were three- to sixfold more potent in antiproliferative activity and three to fivefold more potent in antiangiogenic activity compared to 2ME2. Representatives of the most potent analog families were tested via cassette dosing for PK properties. The formamide, amine and carboxamide analogs all had greater bioavailability compared to 2ME2 when dosed orally. Based on potential toxicity liabilities and intrinsic activities, three carboxamides were selected for further PK evaluation in an oral single-dose CD1 mouse study. All carboxamides had significantly better PK properties (C_{max}, AUC and $t_{1/2}$) compared to 2ME2 in this model. Based on pharmacological evaluation presented elsewhere, carboxamide **9d** is currently in Phase 1 clinical trials for patients with solid tumors under the name ENMD-1198.

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