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# Synthesis and biotesting of new carrier prodrugs of 2-methoxyestradiol

**Natalia A. Lozinskaya,*[a,b] Natalia A. Maximova,[a] Daniil R. Bazanov,[a] Sergey E. Sosonyuk,[a] Birgit Wobith,[c] Nikolay A. Zefirov,[a,b] Elena V. Kharitonashvili,[a] Olga N. Zefirova,[a,b] Sergey A. Kuznetsov[c] and Marina V. Proskurnina[a]**

[a] *Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation. E-mail: natalylozinskaya@mail.ru*

[b] *Institute of Physiologically Active Compounds, Russian Academy of Sciences, 142432 Chernogolovka, Moscow Region, Russian Federation*

[c] *Institute of Biological Sciences, Department of Mathematics and Natural Sciences, University of Rostock, D-18059 Rostock, Germany*

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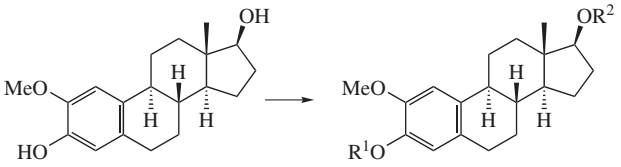
**To overcome the metabolic instability of anticancer agent 2-methoxyestradiol, two its dichloroacetylated prodrugs and three new control compounds have been synthesized and evaluated in different *in vitro* assays with cancer cells A549 and MCF-7. Both prodrugs demonstrated an intrinsic cytotoxicity to A549 cells with no effect on the cellular microtubule network. Molecular modeling has revealed putative weak halogen bond formation of 17-O-dichloroacetylated 2-methoxyestradiol with GTP in the α-tubulin subunit.**

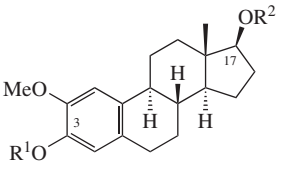



2-methoxyestradiol — 15–97%, toxic to cancer cells, $EC_{50}$ 1.5–17.9 μM

$R^1$ = H, $C(O)CHCl_2$, $C(O)CH_2Cl$; $R^2$ = $C(O)CHCl_2$, $C(O)CH_2Cl$

*Keywords*: 2-methoxyestradiol, dichloroacetate, anticancer, tubulin.

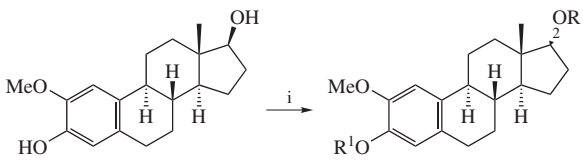
2-Methoxyestradiol (2-ME) **1a** is an endogenous metabolite of estrogen hormone, which has promising antitumor activity and relatively low toxicity to normal cells at therapeutically effective doses.[1–3] However, its rapid metabolism affecting both hydroxyls hampers the clinical application. One way to overcome the metabolic instability of compound **1a** and its analogues is to synthesize their C-17 and/or C-3 ester prodrugs. Modification at the C-3 and C-17 atoms of 2-ME usually, with rare exceptions, leads to a decrease in anticancer activity;[3] therefore, the corresponding prodrugs are typically inactive or little active *in vitro*, but undergo conversion to the initial molecule *in vivo*. For example, the phosphate ester analogue **1b**, bearing the acetylated hydroxyl group at the C-17 atom to block metabolism, is less active than compound **1a** against cancer cells, but is more effective in the treatment of Barrett's esophageal adenocarcinoma xenograft in mice.[4]




**1a** $R^1 = R^2 = H$
**1b** $R^1 = CH_2OP(O)(OH)_2$, $R^2$ = Ac

In this work, we suggested dichloroacetate (DCA) as the ester group for the metabolically blocked prodrugs of compound **1a** (Scheme 1, compounds **1c**,**d**). The choice was based on the intrinsic weak anticancer activity of DCA itself, namely stimulation of apoptosis in malignant cells at high doses, as well as its lowest toxicity to normal cells.[5,6] However, the question arises whether the synthesized esters are stable in the cells, because the hydrolysis rate for the prodrugs should be low. Chlorine atoms in compounds **1c** and **1d** can influence the ester hydrolysis by cellular esterases in two opposite directions, namely the electron withdrawing properties promote the hydrolysis, while the bulkiness of substituents hinders the access for the metabolic enzymes. To check the stability of prodrugs **1c** and **1d**, we synthesized them and tested *in vitro* along with control compound **1e** bearing monochloroacetyl moiety.

Esterification of 2-methoxyestradiol **1a** with an equivalent amount of dichloroacetyl chloride in the presence of 4-(dimethylamino)pyridine (DMAP) resulted in a mixture of mono- and diesters **1c** and **1d**, respectively, which proved to be unstable on silica gel and were separated by careful crystallization (see Scheme 1). In $^1$H NMR spectra of compounds **1c** and **1d**, the signal of proton at the C-17 atom is shifted downfield compared with that of compound **1a** and is observed at 4.82 and 4.84 ppm, respectively (see Online Supplementary Materials). The formation of the second ester moiety in compound **1d** is confirmed by both the absence of




**1a**

**1c** $R^1$ = H, $R^2 = C(O)CHCl_2$, 15%
**1d** $R^1 = R^2 = C(O)CHCl_2$, 81%
**1e** $R^1 = R^2 = C(O)CH_2Cl$, 97%

**Scheme 1** *Reagents and conditions*: i, $Cl_2CHC(O)Cl$ (for **1c** and **1d**) or $ClCH_2C(O)Cl$ (for **1e**), DMAP, $CH_2Cl_2$, room temperature, 24 h.

**Table 1** Results of biotests for the synthesized compounds **1c–e** and **2b**,**c**.

| Compound | A549 cells growth inhibition at 10 μM (%) | Cytotoxicity to MCF-7 cells, ($EC_{50}$/μM) | Effect on MT in A549 cells at 10 μM | Effect on MT in A549 cells at 100 μM | Effect on A549 cells shape and attachment at 100 μM |
|---|---|---|---|---|---|
| **1c** | 77 | 17.9 | No effect | Weak effect, MT curling in some contracted cells | Cell contraction and strong cell detachment |
| **1d** | 75 | 2.7 | No effect | Weak effect, MT curling in some contracted cells | Cell contraction and strong cell detachment |
| **1e** | 80 | 1.5 | Complete depolymerization of MT in ~80% cells | Complete depolymerization of MT in all cells | Cell rounding and strong cell detachment |
| **2b** | n.d.[a] | >50 | n.d. | n.d. | n.d. |
| **2c** | n.d. | >50 | n.d. | n.d. | n.d. |
| **1a** (2-ME) | 100 | 1.2[b] | Complete depolymerization of MT | n.d. | n.d. |
| **2a** (estrone) | n.d. | 40.3 | n.d. | n.d. | n.d. |

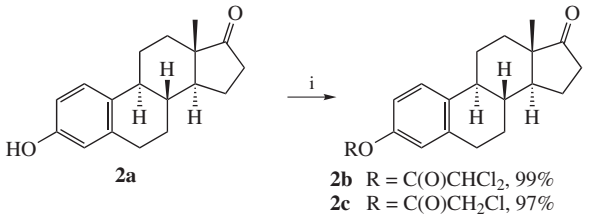
[a] n.d. – not determined. [b] $EC_{50}$ value obtained in this work was in accordance with the known data.[7]

the singlet of phenolic hydroxyl and the appearance of two $CHCl_2$ proton singlets at 6.19 and 5.97 ppm. An analogous procedure was used for the synthesis of control compound **1e**, which was obtained in excellent yield (see Scheme 1). The signal of proton at the C-17 atom in $^1$H NMR spectrum of chloroacetic acid ester **1e** was observed at 4.77 ppm.

Compounds **1c–e** were tested *in vitro* for their ability to inhibit the growth of human lung carcinoma cells A549 using microscopy for direct cell counting over 24 and 48 h of culturing. As it was formulated above, we had reasons to expect much less activity of the stable ester prodrugs compared with the parent drug **1a**. However, the esters revealed a significant antiproliferative effect (Table 1).

The following evaluation of the synthesized compounds in a standard colorimetric MTT assay (see Online Supplementary Materials) with human breast cancer cell line MCF-7 indicated, that cytotoxicity was in accordance with the above data on the cell growth inhibition for diesters **1d** and **1e**, while monoester **1c** was one order of magnitude less cytotoxic (see Table 1).

To confirm that the activity of diesters **1d**,**e** did not originate from the activity of DCA formed due to easily hydrolyzable phenolic ester bond, we synthesized and tested phenolic esters of noncytotoxic estrone **2a**, namely compounds **2b**,**c** (Scheme 2). Indeed, as it is seen from Table 1, both dichloroacetic and chloroacetic acid esters of estrone were inactive against MCF-7 cells in the MTT




**2a**

**2b** R = $C(O)CHCl_2$, 99%
**2c** R = $C(O)CH_2Cl$, 97%

**Scheme 2** *Reagents and conditions*: i, $Cl_2CHC(O)Cl$ (for **2b**) or $ClCH_2C(O)Cl$ (for **2c**), DMAP, $CH_2Cl_2$, room temperature, 24 h.

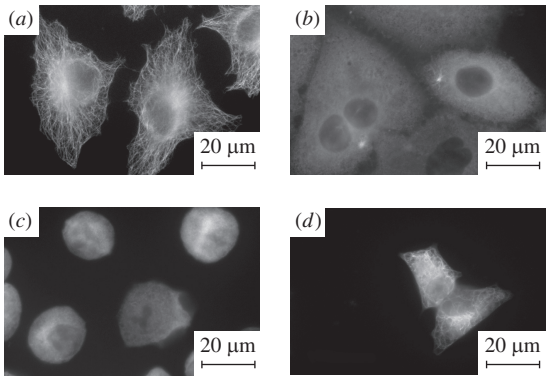



**Figure 1** Immunofluorescence microscopy images of MT in human lung carcinoma A549 cells: (*a*) intact MT in 0.5% DMSO; (*b*) depolymerized MT in 10 μM solution of compound **1e**; (*c*) rounded cells without MT in 100 μM solution of compound **1e**; (*d*) contracted cells with MT curling in 100 μM solution of compound **1c**.

test. Thus, the activity of compounds **1d**,**e** in both cell growth assay and MTT test can be rationalized either by their intrinsic cytotoxicity or by the activity of 2-methylestradiol **1a** released due to rapid hydrolysis of both ester moieties.

To understand the rationale, we investigated the effect of compounds **1c–e** on microtubules (MT) dynamics in A549 cells using immunofluorescence microscopy, since 2-methoxyestradiol **1a** is known to cause disassembly of MT [see Table 1 and Figure 1(*a*),(*b*)]. No effect on MT was observed for DCA esters **1c** and **1d**, while monochlorinated ester **1e** caused depolymerization of MT as well as cell rounding and detachment [see Table 1 and Figure 1(*b*),(*c*)]. These results support the origin of antiproliferative properties for compound **1e** from rapid hydrolysis of both chloroacetic groups inside the cells, while DCA esters **1c**,**d** are more stable, at least considering the C-17 site. Compounds **1c**,**d** did not promote the MT depolymerization, instead they caused cell contraction, detachment and finally an MT curling at 100 μM concentration [see Table 1, Figure 1(*d*)]. The last effect differs from an unusual MT curling observed for some 2-methoxyestradiol and podophyllotoxin derivatives in our previous works,[8–11] and is most probably caused by the damage of MT connection to centrosome, resulting in MT gliding and curling.[12] Hence, the immunofluorescence microscopy data further confirm the intrinsic cytotoxic activity of DCA esters **1c** and **1d**, which is probably related to their interaction with molecular target differing from the colchicine binding site in tubulin.

This conclusion is in accordance with the following molecular modeling data. Molecular docking of compound **1c** into the 3D model of the colchicine binding site in tubulin (PDB ID: 4O1d) using an AutoDock Vina 1.1.2 software[13] and an USCF Chimera 1.13.1 visualization system[14] indicates that for all ligand–protein complexes with the best scoring functions, the position of the compound **1c** steroid core differs significantly from the 2-methoxyestradiol **1a** position according to the classic pharmacophoric model of a ligand for the colchicine binding site[15] [Figure 2(*a*)]. Note that one of the chlorine atoms is exposed towards the GTP site in α-subunit [Figure 2(*b*)] and can form a weak halogen bond (3.4 Å, 155.6°) with the phosphate oxygen atom in GTP, the examples of such halogen bond formation with nucleotide phosphate oxygen atom as an electron donor have been recently reviewed.[16]

In summary, the results of the *in vitro* biotests have revealed that DCA prodrugs of 2-methoxyestradiol possess an intrinsic cytotoxicity to A549 cells. However, they do not inhibit MT polymerization, which confirms their relative stability in the cells and makes them promising compounds for further biotesting *in vivo*.

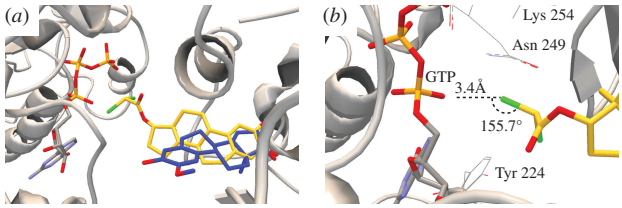



**Figure 2** Molecular docking results: (*a*) location of the conjugate **1c** (yellow) and 2-methoxyestradiol (blue) in α,β-tubulin dimer, as predicted by automated docking using AutoDock Vina 1.1.2, α-subunit is presented at the left side and β-subunit at the right side, hydrogen atoms are omitted for clarity; both oxygen atoms of the ester moiety at the C-17 atom can form hydrogen bonds with Lys 254(β), Tyr 224(α) and Asn 249(α); (*b*) putative halogen bond between chlorine atom and the oxygen atom of GTP is indicated by dashed line.

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*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.01.002.

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