2.2.2. Cell proliferation assays

The cell proliferation assay was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Cell Titer 96 Aqueous, Promega, Nepean, ON, Canada) as previously described [4, 22]. Briefly, cells were plated in triplicate in 96-well plates (1 x 10^4 cells/well) in appropriate culture medium (total of 90 µL). Before each treatment, the cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 24 h. The different aminosteroids were dissolved in methanol (50 mM). The stock solutions were diluted at multiple concentrations with culture media to obtain the desired final concentration by adding 10 µL in each well, and the mixture incubated for three days. Following treatment, 10 µL of a solution of MTS were added to each well and the mixture was incubated for 4 h. The plates were subsequently analyzed at 490 nm using a Tecan M-200 microplate reader (Männedorf, Switzerland) and the % of cell proliferation (100% for the control) and the % of cell growth inhibition (0% for the control) were calculated. For mestranol derivatives **23**, **48** and RM-581, IC_{50} values (50% of cell growth inhibition) were calculated using GraphPad Prism 6 software [25].

3. RESULTS AND DISCUSSION

3.1. Solid-phase synthesis of mestranol derivatives

To generate different libraries of RM-581 analogues, we had to prepare its basic core, represented by compound **1**, and then load it on the polystyrene diethylbutylsilane (PS-DES) resin for SPOS. Compound **1** was obtained from commercially available estrone (E1) in four steps with a global yield of 21% (**Scheme 1**). In the first three steps, E1 was iodated to provide 2-iodo-E1 [21], the 3-OH was protected as a methoxy via a methylation in basic medium with methyl iodide and the substitution of the iodine by a piperazine core was performed by Ullmann reaction. This step of the synthesis was problematic because it gave low yields (approximately 40%) but we succeed to increase the yield to 68% by using a large excess of piperazine (40 eq.). Finally, compound **1** was obtained following the introduction of an ethynyl group at position 17α of 2-piperazino-3-methoxy-E1. This transformation was performed in two steps with trimethylsilylacetylene in presence of methyl lithium and by a subsequent basic hydrolysis of the trimethylsilyl group giving compound **1**.

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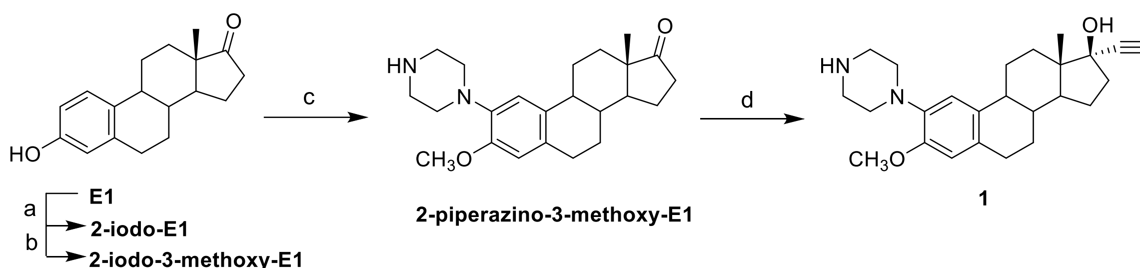
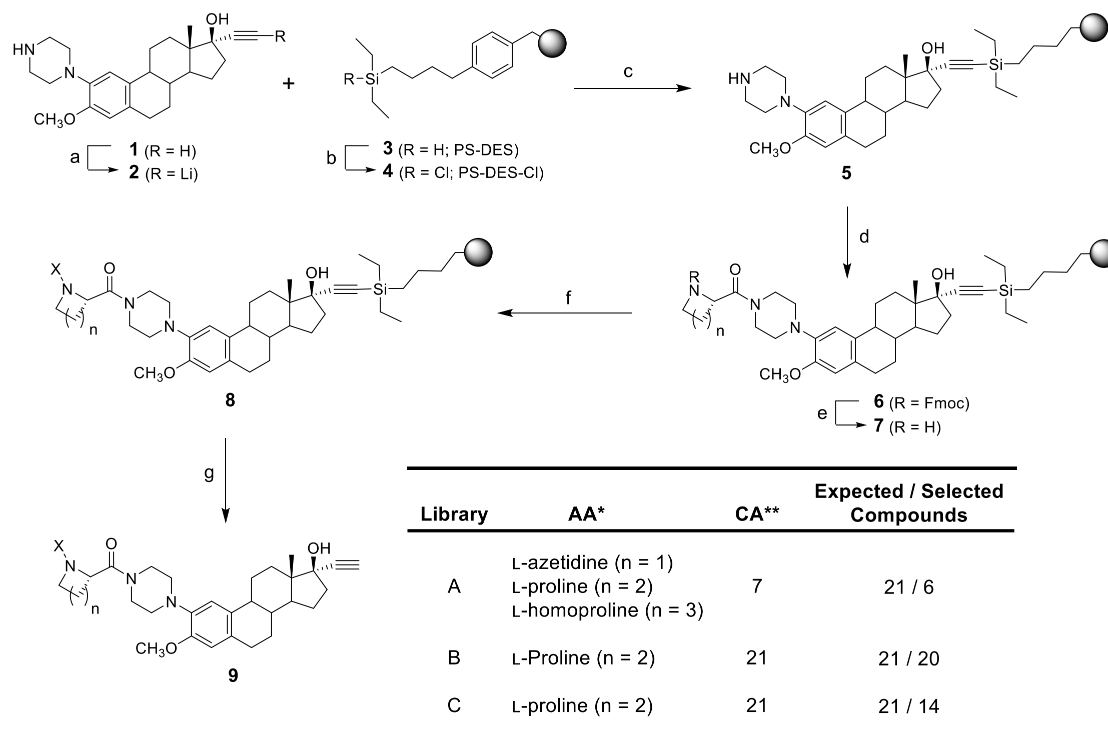


Scheme 1. *Reagents and conditions:* (a) $Hg(OAc)_2$, I_2, AcOH/THF, rt, 2 h; (b) MeI, Cs_2CO_3, acetonitrile, reflux, 2.5 h; (c) piperazine, K_2CO_3, CuI, L-proline, DMSO, 120 °C, overnight; (d) i. TMS-acetylene, MeLi, THF, rt, overnight; ii. K_2CO_3, MeOH, rt, 5 h.

The same conditions reported by our research group for the loading of the C19-steroid core of RM-133 were used to perform the loading of compound **1** (a C18-steroid) on PS-DES resin **3** (loading capacity of 1.47 mmol/g) (**Scheme 2**) [19, 20]. The global loading previously reported by our group was about 50%, while this time we obtained a lower average loading of 25%. The reason for a lower coupling yield was due to the poor solubility of the organolithium **2** in THF, thus hindering the addition and the loading of this steroid derivative to the chlorosilyl resin **4**, previously generated *in situ* from PS-DES resin **3**. We succeeded in improving the solubility of the organolithium by adding a small amount of DMSO but a too large volume of this solvent would interfere with this loading reaction. We tried distinct combinations of THF and DMSO and different total volumes but we could not exceed a maximum of 36% of loading after several assays. However, steroid derivatives which have not been loaded onto the resin can be recovered and used again. Thus, a series of loading reactions were necessary to obtain enough resin **5** (~ 15 g) to generate many RM-581 analogues by SPOS.

Equal portions of resins **5** were then placed in each well-reactor to introduce the first level of molecular diversity via a peptide coupling of resins **5** using different amino acids (AA) to provide resins **6**. For this first series of RM-581 analogues, three protected amino acids (L-azetidine-Fmoc, L-proline-Fmoc and L-homoproline-Fmoc) were selected based on previous structure-activity relationship (SAR) results obtained with the C19-steroid core of RM-133 [19]. The Fmoc-protecting group of resin **6** was then removed by a treatment with piperidine in DMF and, after washing resins **7**, the second level of molecular diversity was introduced by a peptide coupling of resins **7** with different carboxylic acids (CA) to give resins **8**. The final step of SPOS required an acid cleavage to generate the free mestranol

derivatives **9**. Seven carboxylic acids were selected and used for this first series of aminosteroid derivatives for a total of 21 compounds (3 AA x 7 CA) (see **Table S1**).



Library	AA*	CA**	Expected / Selected Compounds
A	L-azetidine (n = 1) L-proline (n = 2) L-homoproline (n = 3)	7	21 / 6
B	L-Proline (n = 2)	21	21 / 20
C	L-proline (n = 2)	21	21 / 14

Scheme 2. Solid-phase synthesis of ethynylated aminosteroid derivatives using the key diethylsilyl acetylenic linker. *Reagents and conditions:* (a) MeLi, THF-DMSO 8:2, 0 °C to rt, 1.5 h; (b) 1,3-dichloro-5,5-dimethylhydantoin, DCM, rt, 1 h; (c) THF, rt, overnight; (d) L-azetidine-Fmoc, L-proline-Fmoc or L-homoproline-Fmoc, HBTU, DIPEA, DMF, rt, 4 h (2 coupling cycles); (e) piperidine 20%, DMF, rt, 1 h; (f) X-COOH, HBTU, DIPEA, DMF, rt, 3 h; (g) HCl, MeOH, DCM, rt, overnight.

From the HPLC purity analysis of all the aminosteroid derivatives of library A, we observed a trend related to the AA; indeed, an average HPLC purity of 40% was measured for compounds with L-azetidine as AA. On the other hand, compounds **9** with L-homoproline as AA showed very low purities (~ 5%) or no coupling. Nevertheless, six of the seven compounds with L-proline as AA showed an acceptable HPLC purity (> 70%) and were thus selected for biologigal assays (compounds **10-15**, Fig. **2**). These results reflect the reaction variability of these three AA depending on the reagents and conditions used for the peptide coupling.

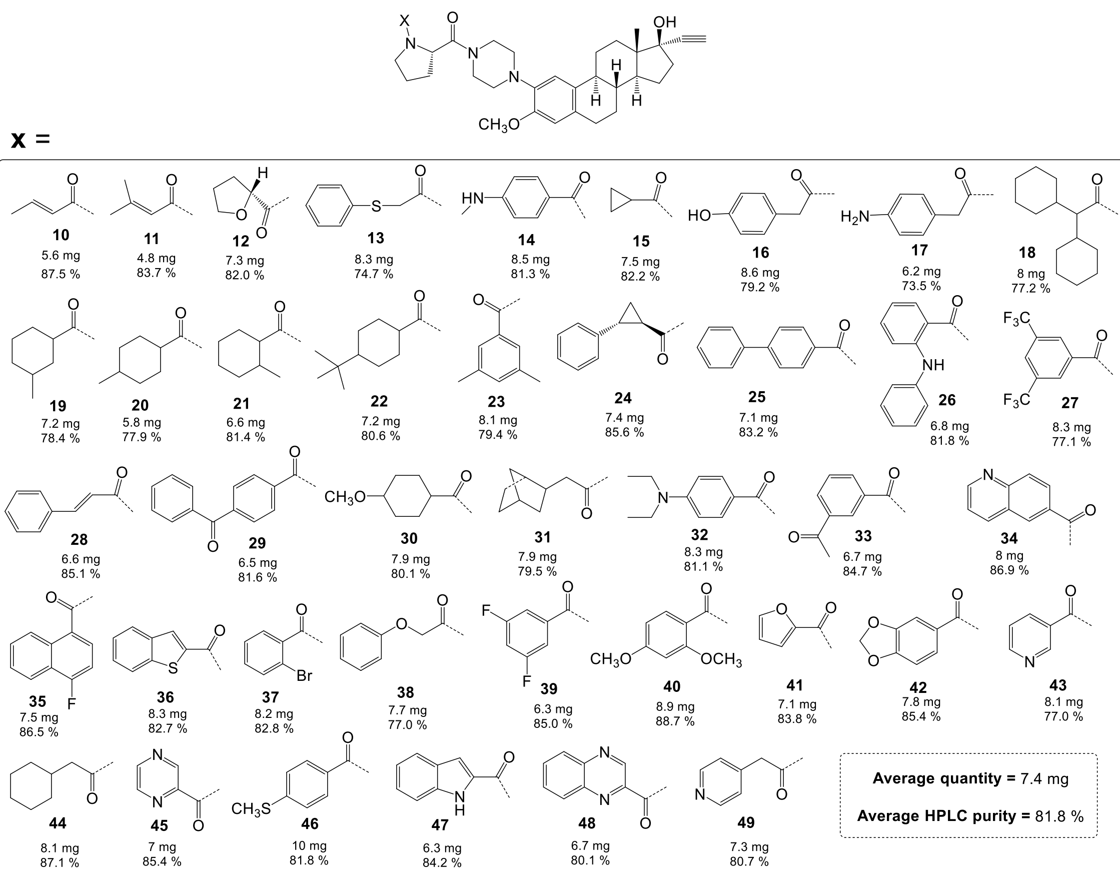


Fig. (2). Compound number, quantity (mg) and HPLC purity (%) of RM-581 analogs represented by mestranol derivatives **10-49**.

Based on these results, we decided to focus on the second level of molecular diversity by the introduction of a diversity of CA residues (X) and to retain only the L-proline as AA for the synthesis of the next aminosteroid derivatives **9**. Thereby, two other libraries (B and C) of aminosteroid derivatives with this AA and 21 different CA in each case (see Supporting Information, **Table S1**) were then generated using the same conditions as for the synthesis of library A. HPLC purities of 20 library-B products from the 21 members showed higher than 70% and were thus selected for biological assays (compounds **16-35**, Fig. **2**). For the library C, 14/21 products showed acceptable purities to be selected for biological assays (compounds **36-49**, Fig. **2**). Globally, a total of 40 mestranol derivatives (compounds **10-49**) with L-proline as AA and 40 different X groups were selected for a first round of biological screening. Their structures, recovered quantities and HPLC purities are reported in Fig. **2**.

3.2. Antiproliferative activities (screening study) of mestranol derivatives 10-49

The antiproliferative activities of RM-581 analogs **10-49** were evaluated on a wide range of cancer cell lines (Fig. **3**) using a MTS proliferation assay [22]. We selected nine cancer cell lines representative of four cancers: two among the most common diagnosed (breast and prostate cancers) and two with a bad prognosis (ovarian and pancreatic cancers). All compounds were tested at two concentrations and the results were expressed as the percentage of cell growth inhibition (100% basal cell proliferation) (see Supporting Information, **Table S2**). RM-581 was used as a positive reference for all these assays [13]. To simplify the reading of these cancer cell viability results, we also reported the cytotoxic activity of compounds **10-49** in terms of cell growth inhibition at a single concentration (Fig. **3**). Based on these first biological results, we first observed that the most active candidates were the same, independently of the cancer cell lines tested; this is the case of compounds **23**, **28**, **31**, **39**, **42**, **47** and **48** which have shown interesting anticancer activities on T-47D, MCF-7, LAPC4, Caov-3, PANC-1 and BxPC-3 cell lines. This tendency is very interesting, pointing out to a common molecular checkpoint targeted by these analogs, regardless of the cancer cell line's nature. Although a little less active, compounds **19**, **21**, **35**, **36**, **38** and **44** have also shown significant antiproliferative activity on T-47D, MCF-7, LAPC4, Caov-3 and PANC-1 cells. Furthermore, compounds **13**, **20**, **24**, **41**, **45** and **46** exhibited good anticancer properties against T-47D and LAPC4 cancer cells.

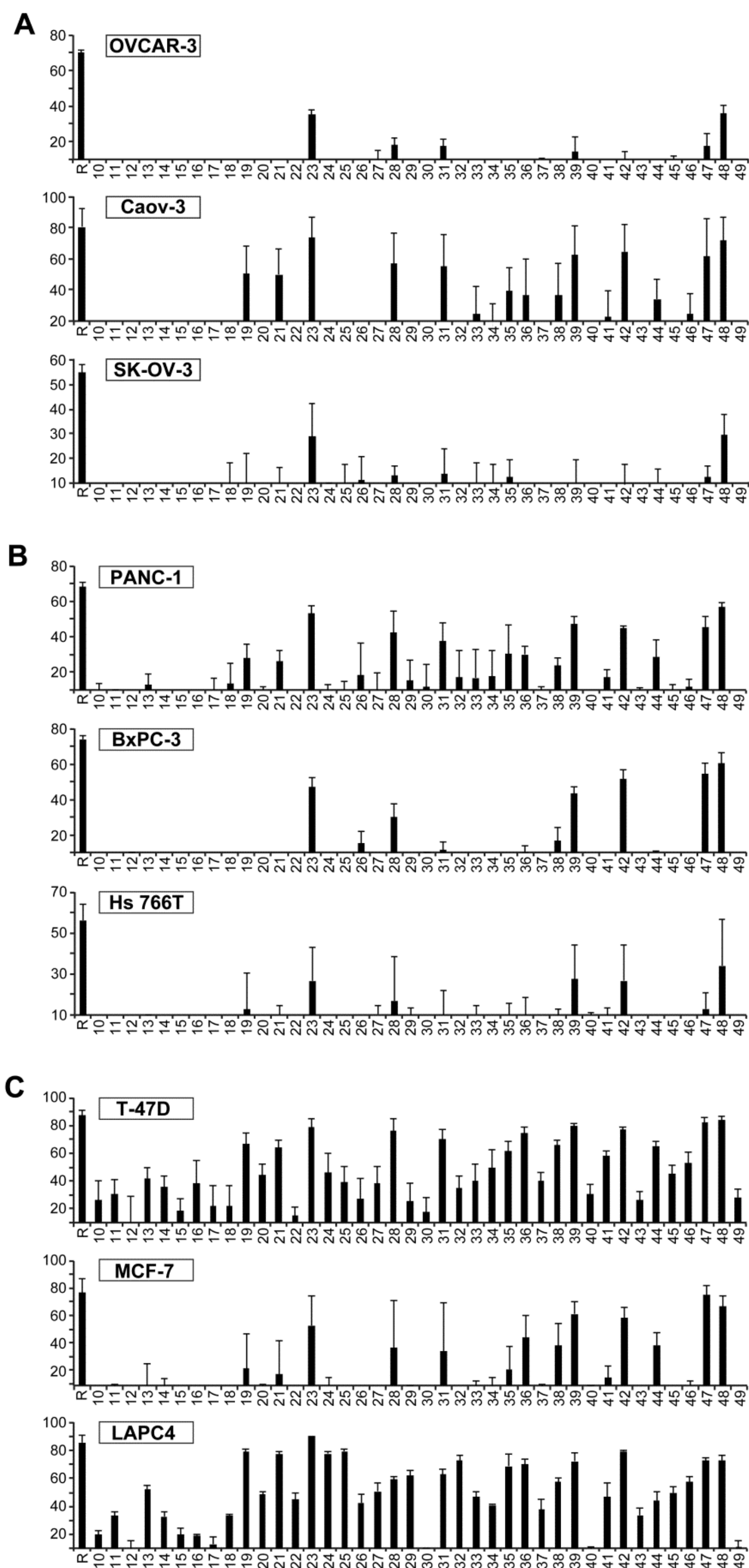
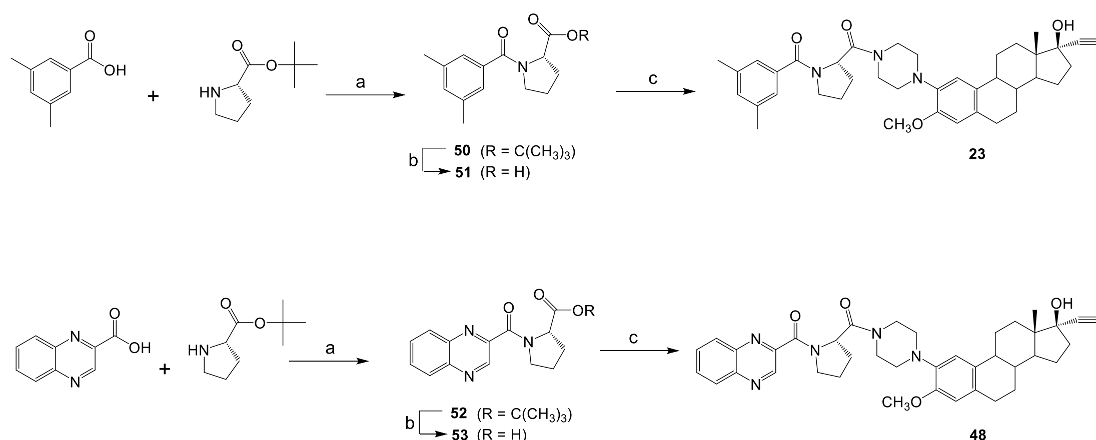


Fig. (3). Cell growth inhibition (%) of nine cancer cell lines by mestranol derivatives **10-49** and RM-581 (R) at a concentration of 5 μM. Cell lines are representative of different cancer types: ovarian (**A**), pancreatic (**B**) and breast/prostate (**C**). Results lower than 10% were not shown.

In light of these biological results, we cannot identify a clear SAR, but derivatives with hydrophobic X groups seemed to be better cytotoxic agents. Another important observation is that all biologically active compounds are less potent than the reference RM-581 for all the tested cell lines. However, it should be emphasized that the anticancer activity of compounds **10-49** is probably higher than that observed, because they are pure only to about 80% contrary to reference compound RM-581, which is pure at 99.6%. Otherwise, aminosteroids **23** and **48** have shown a cytotoxic activity that is very like that of RM-581 on all tested cancer cell lines. Regarding compound **48**, this is not surprising, considering that its chemical structure is very close to that of RM-581 with a quinoxaline moiety as X group instead of a quinoline (Fig. **1** and **2**). Nevertheless, biological results obtained with compound **23** are particulary interesting, because its X group (3,5-dimethylbenzoyl) is smaller than that of compound **48** and RM-581. As lead candidates, compounds **23** and **48** were therefore selected to determine their IC_{50} values on several cancer cell lines in comparison to RM-581, but before, they were synthesized in solution-phase organic synthesis, instead of SPOS, purified, and fully characterized.

3.3. Synthesis of mestranol derivatives 23 and 48 in solution

Compounds **23** and **48** were synthesized by classic chemistry as shown in **Scheme 3**. The left part of the side chains of these two compounds was first obtained by a peptide coupling between L-proline *tert*-butyl ester and the corresponding carboxylic acid (3,5-dimethylbenzoic acid and quinoxalinecarboxylic acid respectively for compounds **23** and **48**) using the conditions described previously for SPOS. The *tert*-butyl esters **50** and **52** were then treated with trifluoroacetic acid (TFA) to generate corresponding acids **51** and **53**. Finally, compounds **23** and **48** were obtained via a peptide coupling of **51** and **53**, respectively, with the piperazine moiety of the steroid core **1**. After a purification step by silica gel chromatography, the HPLC purities of final compounds **23** and **48** were found to be 100.0% and 95.0%, respectively. As well as for RM-581 [13] and RM-133 [8], two rotamers were observed in ^{1}H and ^{13}C NMR spectra for compound **48,** but this effect was clearly less pronounced for compound **23**, showing a single major rotamer. In addition to NMR, IR and mass analyses supported the expected structures.



Scheme 3. *Reagents and conditions:* (a) HBTU, DIPEA, DMF, rt, overnight; (b) TFA-DCM (95:5), rt, 3 h; (c) Compound **1**, HBTU, DIPEA, DMF, rt, overnight.

3.4. Antiproliferative activities (IC_{50} values) of aminosteroid derivatives 23 and 48

Resynthesized and purified compounds **23** and **48** were evaluated on several cancer cell lines in comparison with RM-581 (**Table 1**). Considering these results, we can see a very interesting gain in antiproliferative activity of compounds **23** and **48** in comparison with the first biological screening in which their HPLC purities were lower with an average purity of ~80% (Fig. **2**). Indeed, compound **48**, with a side chain very like that of RM-581, showed a cytotoxic activity slightly higher than that of RM-581 in almost all cancer cell lines. However, compound **23** has shown an anticancer activity that is about twice as high as that of RM-581 on PC-3, LNCaP, MCF-7, OVCAR-3 and PANC-1 cells. Otherwise, the antiproliferative activities of RM-581 and compound **23** are very close on LAPC4 and T-47D. This major gain in activity of compound **23** is particulary interesting, because its X group is smaller with a lower molecular weight, and potentially more chemically stable than the N-heterocycle of RM-581 and compound **48**, which are both potentially sensitive to *in vivo* N-oxidation [23]. Finally, it should be emphasized that, like RM-581, compounds **23** and **48** are active against all the cancer cell lines tested, suggesting a similar mechanism of action acting on a commun biological target common to different types of cancer. However, the exact mechanism of action remains to be confirmed through additional studies, that the same way that it was performed for androstane aminosteroid RM-133 [5].

Table 1. Cell viability assays (IC_{50} values in μM) of mestranol derivatives **23**, **48** and RM-581 on various cancer cell lines.[a]

#	OVCAR-3 (ovarian cancer)	PANC-1 (pancreatic cancer)	T-47D (breast cancer)	MCF-7 (breast cancer)	LAPC4 (prostate cancer)	LNCaP (prostate cancer)	PC-3 (prostate cancer)
23	3.17** ± 0.10	2.47* ± 0.97	0.38 ± 0.34	1.36* ± 0.31	0.65 ± 0.25	0.56* ± 0.30	0.89* ± 0.63
48	4.91 ± 0.16	3.22 ± 0.81	0.41 ± 0.28	2.12 ± 0.38	0.49 ± 0.18	0.77 ± 0.36	1.65 ± 0.73
RM-581	4.96 ± 0.53	3.94 ± 0.86	0.47 ± 0.15	2.55 ± 0.85	0.61 ± 0.25	1.18 ± 0.58	1.63 ± 0.30

[a] Two experiments performed in triplicate; T-test: * $p<0.05$; ** $p<0.01$ from RM-581.

4. CONCLUSION

To extend the SAR study related to a new promising anticancer agent having an estrane core, 40 aminosteroid derivatives closely related to RM-581, compounds **10-49**, were quickly synthesized by SPOS. Their antiproliferative activities were evaluated on a panel of cancer cell lines, in comparison to RM-581. As a first major observation, the same compounds exhibit antiproliferative activities on different cancer cells. This is notably the case of compounds **19**, **23**, **28**, **31**, **39**, **42**, **47** and **48,** which showed interesting anticancer activities on T-47D, MCF-7, LAPC4, Caov-3, PANC-1 and BxPC-3 cells (Fig. **3**). These results therefore suggest a similar mechanism of action of this series of compounds, but it should be emphasized that the biological targets of RM-581 and its derivatives are still unknown. However, biological results did not permit to identify clear SAR, but aminosteroid derivatives with a hydrophobic group X appeared to be better candidates than hydrophilic ones.

These new cell viability results showed that compound **48**, which possesses a side chain that is very close to that of RM-581, was slightly more active than the latter on the seven tested cancer cell lines (**Table 1**). More interestingly, compound **23** was found to be approximately twice as active as RM-581 on PC-3, LNCaP, MCF-7, OVCAR-3 and PANC-1 cells. In addition to its very potent anticancer activity on cell proliferation, compound **23** is particulary interesting, because its side chain is smaller, and with a lower molecular weight,

which could be advantageous for the logP, and potentially more chemically stable than that of RM-581 [24].

In summary, the parallel synthesis methodology reported for mestranol derivatives was efficient, leading to the generation of a diversity of analogues from which two anticancer compounds (**23** and **28**) were identified as highly potent on different types of cancers, notably pancreatic cancer, for which no treatment is currently available.

CONFLICT OF INTEREST

RD declares no conflict of interest, whereas RM, MP, JR, and DP have ownership interests on patent applications and patents related to these families of aminosteroid derivatives.

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SUPPORTING INFORMATION

Carboxylic acids and amino acids used as building blocks for the SPOS synthesis of library members (**Table S1**), cell proliferation results of mestranol derivatives **10**-**49** tested at two concentrations (1 and 5 μM) and expressed in % (± SE) (**Table S2**), ^{1}H NMR spectra of compounds **10**-**49** and additional characterization data (LC-UV/Ms purity, HRMS, IR and ^{13}C NMR) for resynthesized compounds **23** and **48**.

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