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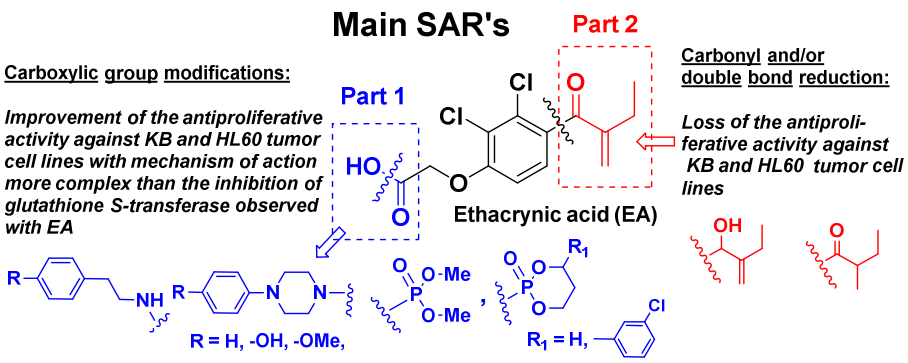
Main SAR's
Part 2
Carboxylic group modifications:
Part 1
Improvement of the antiproliferative activity against KB and HL60 tumor cell lines with mechanism of action more complex than the inhibition of glutathione S-transferase observed with EA
Cl
Cl
O
HO
O
O
Ethacrynic acid (EA)
Carbonyl and/or double bond reduction:
Loss of the antiproli-ferative activity against KB and HL60 tumor cell lines
OH
O
R
NH
R
N
N
O
O-Me
P
O-Me
,
O
O
R1
P
O
R = H, -OH, -OMe,
R1 = H,
Cl

A Novel Class of Ethacrynic Acid Derivatives as Promising Drug-like Potent Generation of Anticancer Agents with Established Mechanism of Action.

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Keywords:

Anti-proliferative activities

Ethacrynic acid derivatives

Apoptosis pathway

Glutathione S-transferase π inhibitors

Abstract: The well-known diuretic Ethacrynic acid (**EA**, Edecrin), showing low anti-proliferative activities, was chemically modified at different positions. The new **EA** derivatives have been tested *in vitro* in anti-proliferative assays on both tumor KB (epidermal carcinoma) and leukemia HL60 (promyelocytic) cells suitable targets for anticancer activity. Reduction of the α-β double bond of **EA** completely abolished anti-cancer activities, whereas introduction of either 2-(4-substituted phenyl)ethanamine (series A) or 4-(4-substituted phenyl)piperazine (series B) moieties generated compounds showing moderate to strong anti-proliferative activities against human cancer cell lines. Several substitutions on the phenyl of these two moieties are tolerated. The mechanism of action of the **EA** derivatives prepared in this study is more complex than the inhibition of glutathione S-transferase π ascribed as unique effect to **EA** and might help to overcome tumor resistances.

Key words: Anticancer agents, ethacrynic acid, ethacrynic acid derivatives, glutathione S-transferase π.

1. Introduction

Cancer is the second leading cause of death in North America and Europe [1]. Although an armada of anticancer agents has received FDA approval over the past two decades [2], the inefficiency of their discovery and development is no longer sustainable and the pipeline of new cancer agents is slim. Today, the outcome of patients with advanced metastasis, for instance regarding lung, colorectal, prostate and breast cancers, remains very poor [3]. The development of anticancer agents for several decades was based on the identification of active compounds, with cytostatic or cytotoxic activity on tumor cell lines, but with many side effects. Consequently, new anticancer agents within new chemical families are urgently needed to allow an extension of this cancer-fighting armada. In addition to the discovery and development of new anticancer agents, an improved understanding of each cancer patient's needs is also essential to enhance the ability of standard treatments to kill cancer cells without significantly affecting normal cells [4].

One important consideration in the development of cancer treatment regimens is resistance against anticancer drugs, which remains a serious obstacle [5]. Indeed, microsomal glutathione transferase 1 (mGST1) and glutathione transferase π (GST π) are often overexpressed in tumors and confer resistance against a number of cytostatic drugs, such as cisplatin and doxorubicin (DOX) [6]. To address this point, the diuretic drug ethacrynic acid (**EA**, Edecrin) **1**, an inhibitor of π class glutathione S-transferase, has been tested against multiple myeloma, and as adjuvant in clinical trials [7]. On the basis of these considerations, Dyson et al [8] and Osella et al [9] synthesized and characterized the bifunctional **EA**-Pt(IV) complex **2** (ethacraplatin) and the **EA**–Pt(II) complex **2'** (cis-diaminobis(ethacrynato)platinum(II) (Figure 1), both of them able to release a cytotoxic platinum (II) agent (inducing apoptosis of cancer cell) and two **EA** moieties (inhibiting glutathione S-transferase (GST) and overcoming drug resistance) via hydrolysis or reduction, respectively. Ethacraplatin **2** resulted to be an excellent inhibitor of GST in several tumor cell lines such as A549 lung adenocarcinoma epithelial cell line, MCF7 and T47D breast tumor cell lines and HT29 colon cancer cell line albeit demonstrating a moderate anti-proliferative activity. Unfortunately, the bifunctional conjugates **2** and **2'** did not offer any advantage over cisplatin for the treatment of malignant pleural mesothelioma (MPM) cells, since the increase of intracellular glutathione (GSH) counteracts the modest inhibition of GST [10]. Recently, Yang et al. constructed biodegradable nanoparticles to codeliver **EA** and dichloro(1,2-

diaminocyclohexane)platinum(II) (DACHPt) which is a precursor of oxaliplatin as a promising approach to overcome the drug resistance in cancer chemotherapy [11]. In vitro studies showed that these hybrid nanoparticles could release both **EA** and DACHPt enhancing of up to ~5 fold of the anticancer efficacy *versus* DACHPt alone. Interestingly, in vivo studies showed better anticancer activities than the simple combination of **EA** and DACHPt.

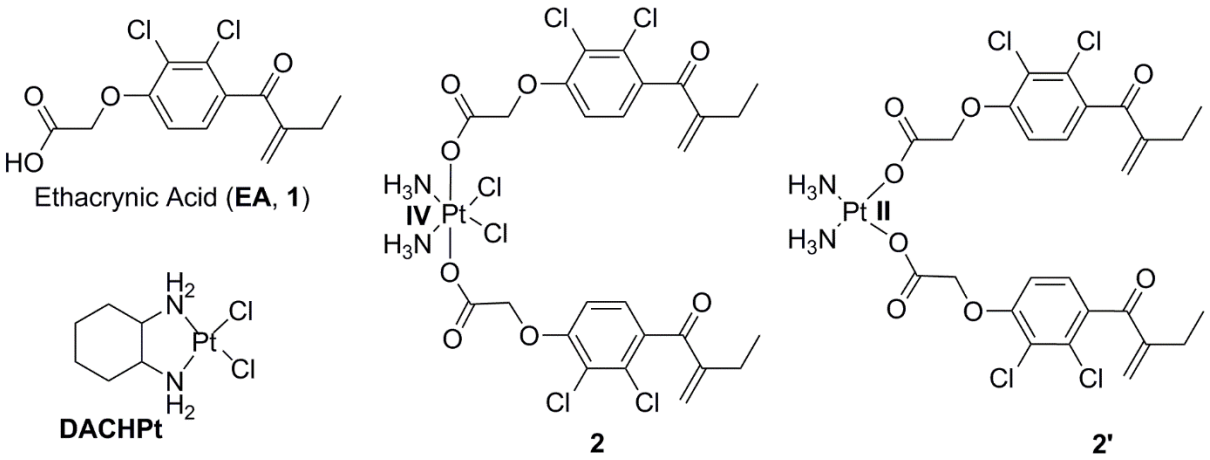


Figure 1.Chemical structure of ethacrynic acid (**EA**, **1**), DACHPt, Pt(IV) and Pt(II) ethacrynic derivatives (**2** and **2'**).

The development of new and potent anticancer agents based on **EA** will undoubtedly offer new opportunities to tackle cancer and some reports in this direction appeared recently reviewed by us and others [12,13]. In order to improve the poor anti-proliferative activity of **EA** [14], we designed original **EA** derivatives based on modifications of the **EA** core structure and tested the resulting derivatives for their capacity to inhibit cell growth in vitro. For this initial screening, we tested the chemicals on three cell lines: two actively dividing cell lines derived from human cancer, human KB carcinoma and human leukemia HL60, and the non-dividing quiescent endothelial progenitor cells (EPC) from Cyprinus carpio. Cell number and cellular NADH content evaluated after 72h chemical exposure provided relevant information concerning viability. The two different tumor cell lines have been selected as representative 'models' of the two common types of cancer: solid tumor cancers (*e.g.* breast, lung, colon, etc.) and blood-based cancers (*e.g.* leukemia, lymphoma, myeloma etc.). According to a recent analysis, the percentage of new patients annually diagnosed for solid tumor cancers and blood-based cancers is 34% and 9%, respectively, whereas the percentage of cancer related deaths is 43% and 9%, respectively [15].

Herein, we report the design, preparation and anti-proliferative activity of original **EA** derivatives active upon cancer cell lines and exemplifying a promising class of potent anticancer agents. The preparation of **EA** derivatives and their synthetic pathways are described in Schemes 1-6 and their mode of action was proposed. The mechanism of action of the **EA** derivatives prepared in this study is more complex that the potent inhibition of π class glutathione S-transferase attributed to **EA** and could potentially overcome tumor resistances.

2. Chemistry

The initial strategic design of these **EA** derivatives can be divided into two parts as shown in Figure 2: part 1: modification of the 3-methylenepentan-2-one unit; and part 2:

modification of the carboxylic acid unit. In this study, we decided not to modify the core of the **EA**.

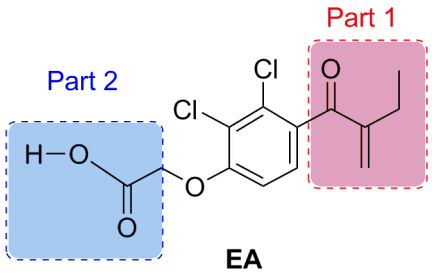
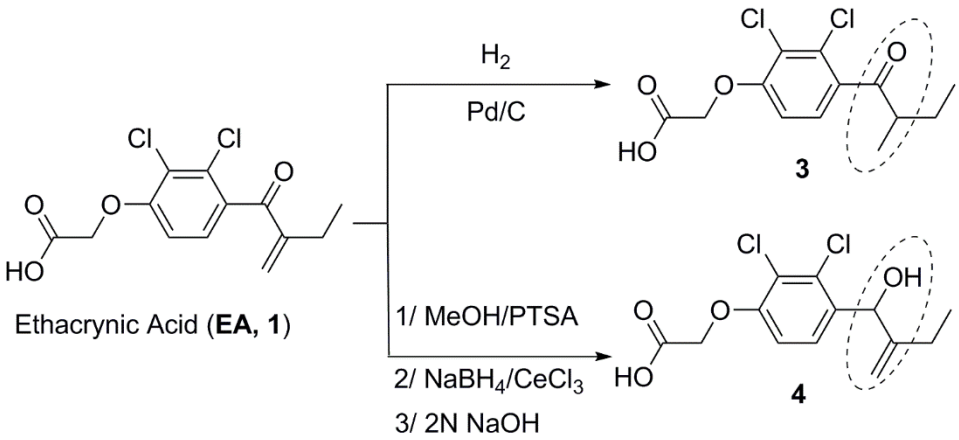


Figure 2. **EA** chemical modifications performed in our study.

As a first strategic attempt to identify a strongly toxic **EA** derivative and to investigate SAR, we targeted the α,β unsaturated carbonyl moiety of **EA** (part 1) as shown in Scheme 1.



Scheme 1.Selective reduction of **EA** to afford **3** and **4**.

The synthesis of compounds **3** and **4** was initiated from the commercially available **EA**. The treatment of **EA** with hydrogen (3 bars) in the presence of Pd/C in isopropanol for 20 min provided compound **3** in quantitative yield. Compound **4** was prepared in three steps. Thus, the treatment of **EA** with methanol in the presence of *p*-toluenesulfonic acid (PTSA) at room temperature led to the corresponding methyl ester. This intermediate was subjected to the Luche reduction with $NaBH_4$ in presence of $CeCl_3$ in a mixture of H_2O/methanol. Then, the reaction mixture was treated with sodium hydroxide in methanol to furnish the free acid **4**.

Then, to further explore SAR in the **EA** series, we directed our efforts to the preparation of two original **EA** derivative series based on modifications in the part 2 (Figure 3): series A (2-(4-substituted phenyl)ethanamine) and series B (4-(4-substituted phenyl)piperazine) and for this purpose **EA** derivatives **5-22** were prepared.

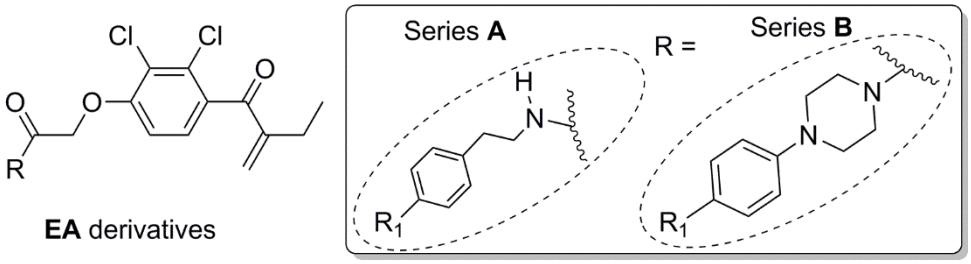
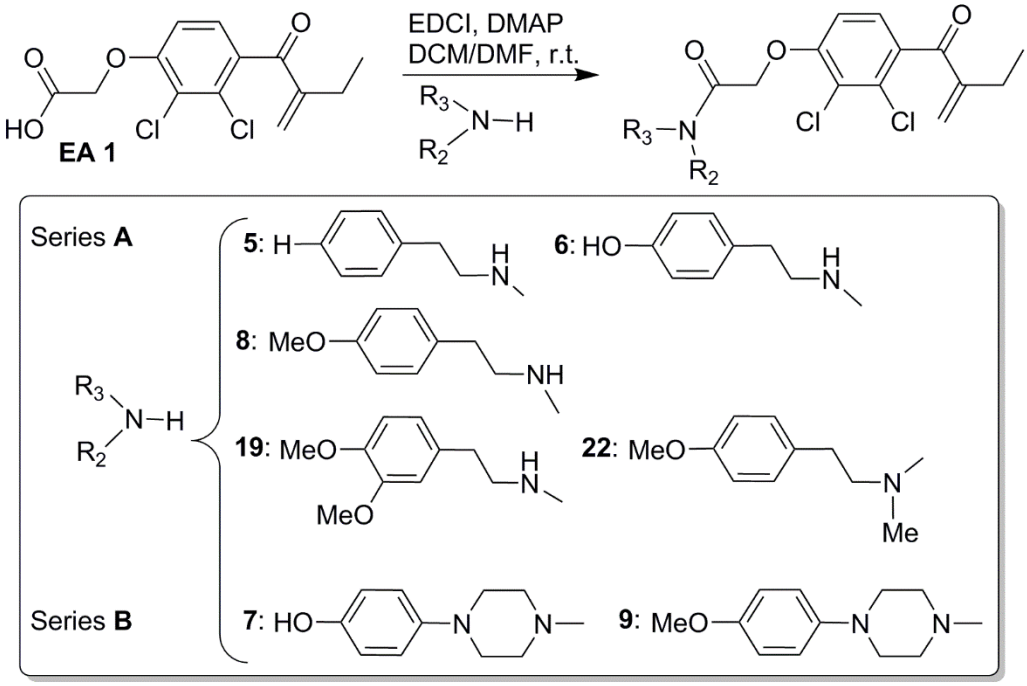
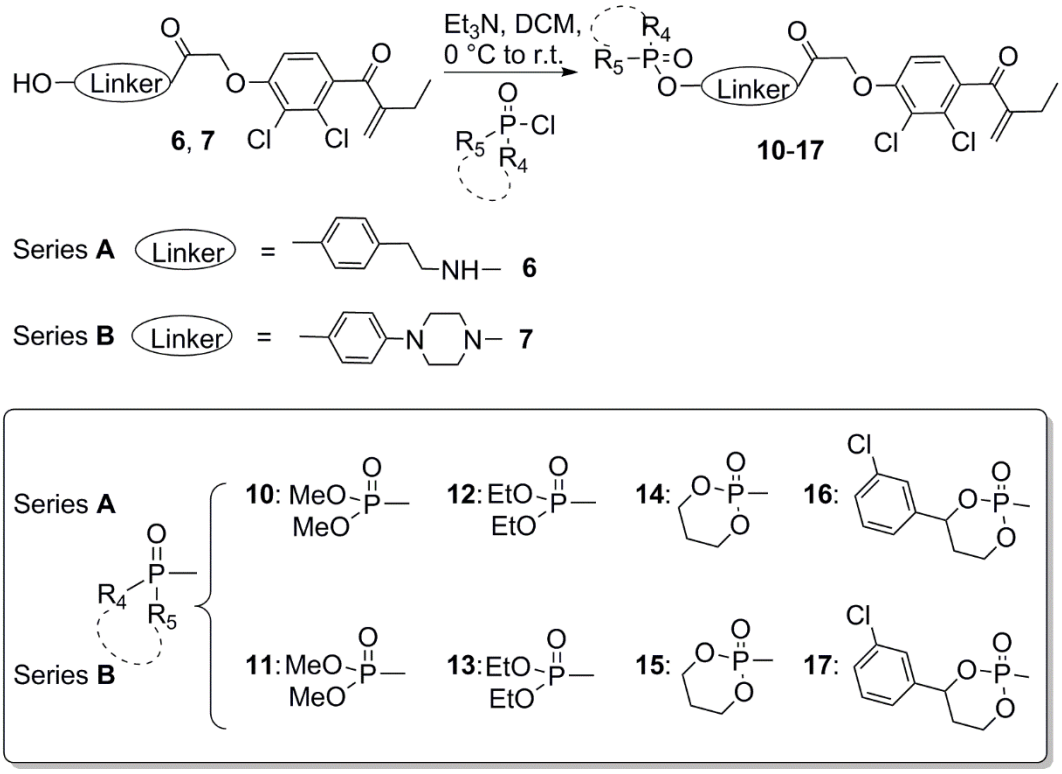


Figure 3. Representation of series A and B developed from **EA**.

The synthesis of **EA** derivatives **5-9**, **19** and **22** is shown in Scheme 2, whereas the synthesis of **10-17** is shown in Scheme 3. The preparation of original derivatives **5-9**, **19** and **22** was achieved in one step synthesis from **EA** and various amines using conventional conditions of peptide synthesis (amines in a mixture of dichloromethane (DCM)/*N*,*N*-dimethylformamide (DMF) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP) at room temperature). The desired products were isolated after flash chromatography in moderate yields (23-25 %). These low yields are essentially due to the formation of by-products of Michael addition on the double bond.



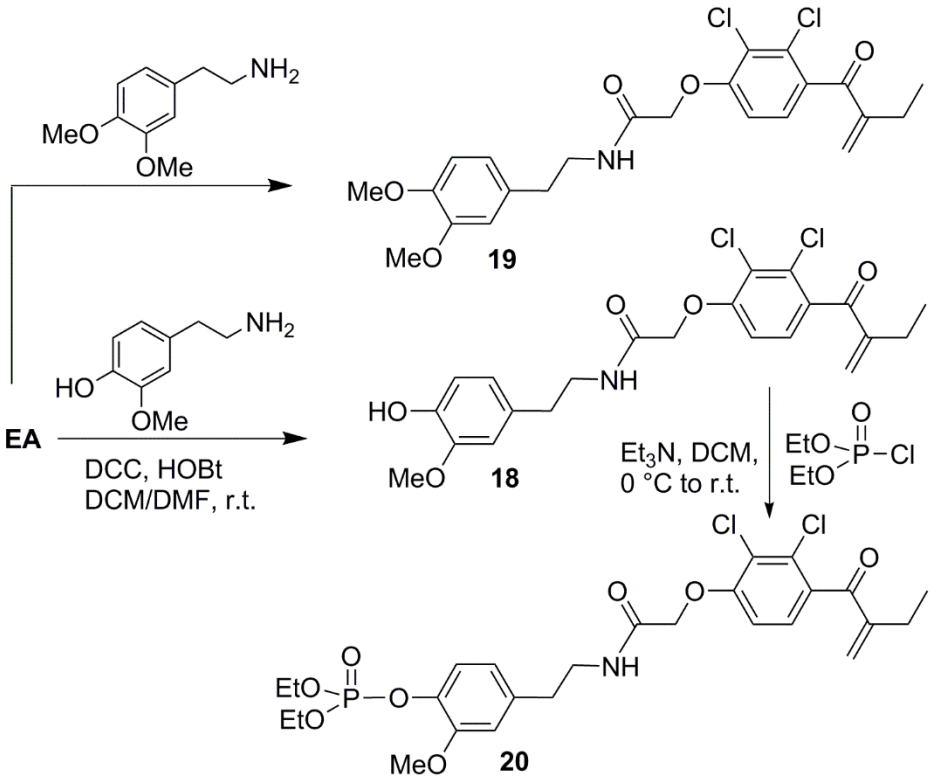
Scheme 2. Synthesis of **EA** derivatives **5-9**, **19** and **22**.



Scheme 3. Synthesis of the phosphate based **EA** derivatives **10-17**.

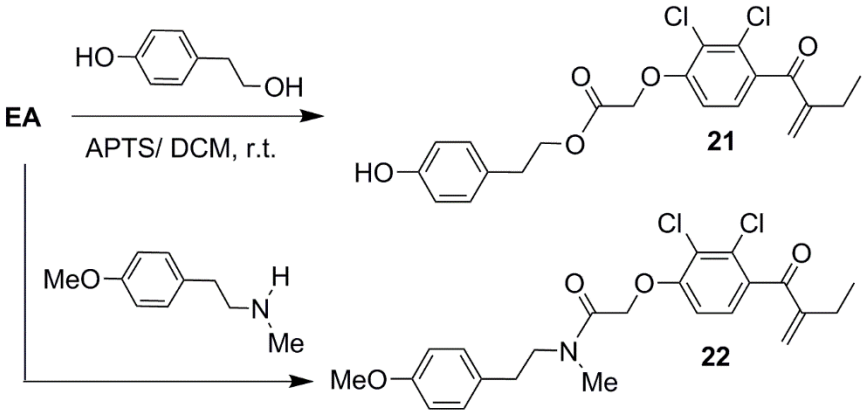
Starting from **EA** containing phenol groups **6** and **7** and either dimethyl chlorophosphate (**10** and **11**), diethyl chlorophosphate (**12** and **13**), 2-chloro-1,3,2-dioxaphosphorinane 2-oxide [16] (**14** and **15**) or 2-chloro-4-(3-chlorophenyl)-1,3,2-dioxaphosphorinane 2-oxide [17] (**16** and **17**) in DCM in the presence of triethylamine, the expected products **10-17** were isolated after purification by flash chromatography in yields ranging between 36 and 67 %.

Based on the very interesting anti-proliferative activities of compounds **8** and **12** (see Table 1, *vide infra*), we decided to prepare the disubstituted analogs **18**, **19** and **20**. Compound **18** was prepared using conventional conditions. Thus, as shown in Scheme 4, **EA** was treated by 4-(2-amino-3-ethyl)-2-methoxyphenol in a mixture of DCM/DMF in the presence of *N*,*N*'-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) at room temperature leading in moderate yield (48%) to the desired compound **18** which in turn was treated by diethyl chlorophosphate in DCM in the presence of trimethylamine to lead to the expected product **20** bearing phosphate moiety in moderate yield (47%). The derivative **19** was obtained from **EA** and 2-(3,4-dimethoxyphenyl)ethanamine using conventional conditions of peptide synthesis (EDCI, DMAP DCM/DMF, r.t.) in 45% yield (see Scheme 2, *vide supra*).



Scheme 4. Synthesis of compounds **18**, **19** and **20**.

Then, to further investigate SAR within the chemical series A we turned our attention to the replacement of the NH group of **6** by either an oxygen or a *N*-methyl group (Scheme 2 and 5). Compound **21** was directly obtained by treatment of **EA** with 4-(2-hydroxyethyl)phenol in the presence of *p*-toluenesulfonic acid (APTS) in dichloromethane (**DCM**) at room temperature with a 92% yield (Scheme 5). Compound **22** was prepared as indicated in Scheme 2 by condensation of **EA** with 2-(4-methoxyphenyl)-*N*-methylethanamine in a mixture of EDCI, DMAP/DCM/DMF, at room temperature with a 92% yield.

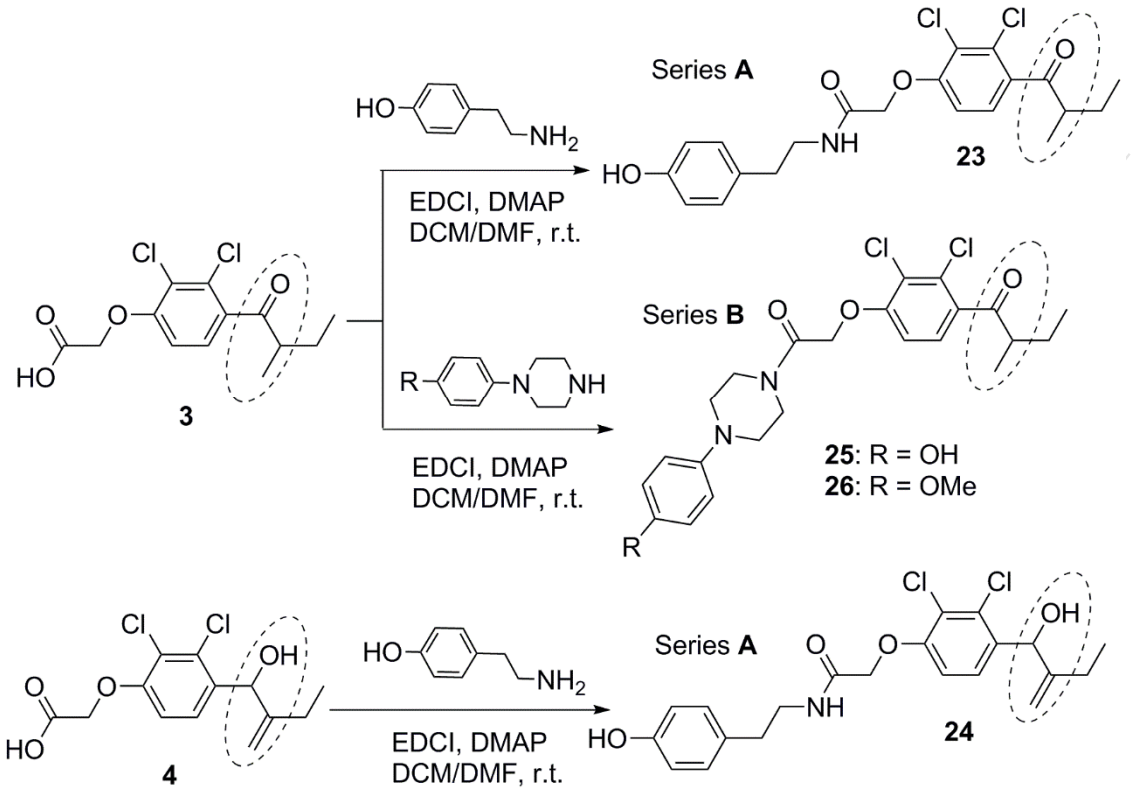


Scheme 5. Synthesis of compounds **21** and **22**.

Finally, our attention has been directed towards the preparation of compounds **23-26** in order to confirm or not the crucial role of the α,β unsaturated carbonyl moiety in the biologically active derivatives **6** (series A), **7** and **9** (series B). The synthetic route leading to compounds **23-26**, described in Scheme 6, was achieved from corresponding reduced **EA** derivatives. Thus, the treatment of **3** or **4** (Scheme 1) in the presence of EDCI and a catalytic amount of DMAP and either 4-(2-aminoethyl)phenol, 4-(piperazin-1-yl)phenol or 1-(4-

methoxyphenyl)piperazine in a mixture of DCM/DMF (Scheme 6) led to the expected products **23** (51% yield), **24** (15% yield), **25** (76% yield) and **26** (75 % yield).

Having in hand a wide range of new **EA** derivatives, we turned our attention to their anti-proliferative properties and to the elucidation of their mechanisms of action.



Scheme 6. Synthesis of compounds **23**, **24**, **25** and **26**.

3. Biological evaluation and discussion

First, based on a MTS (multiple target screening) assay, we examined the *in vitro* anti-proliferative effects of the 26 **EA** derivatives prepared on both KB (epidermal carcinoma) and HL60 (promyelocytic) cells. In addition, we tested these compounds against the non-dividing quiescent endothelial progenitor cells (EPC) to figure a selectivity index (SI) and might suggest that these chemicals are specifically acting on cells with a rapid proliferation.

Selective reduction of the ethylene double bond of **EA** (part 1, Figure 2) afforded the compound **3** and totally abolished the moderate toxicity of **EA**, whatever the cell line exposed (KB and HL60). Similarly, the simple reduction of the carbonyl moiety to the hydroxyl group of **EA** gives the derivative **4,** which was found also inactive.

Few papers described the transformation of the part 1 of **EA**. I. Janser et al. [12k] highlighted the modification on the unsaturated double bond or the phenyl ring of **EA** derivatives but not from **EA** itself. Analogues displayed no or very limited activities either in anti-proliferative or anti-metastatic activities in wound healing assays at concentration ranged 50-100 µM. In another study performed by Cottam et al. [12c], the reduction of the α-β double bond of **EA** led to a compound corresponding to our compound **3**, and completely abrogated the anti-proliferative activity against chronic lymphocytic leukemia (CLL) and the inhibition of the canonical Wnt/β-catenin signaling pathway. Never in these studies the

carbonyl group has been reduced like in our study (compound **4**). It can be concluded that, the reduction of the α-β double bond of **EA** completely abolished anti-cancer activities.

A major urinary metabolite of **EA** results from the condensation of the ethylene double bond to the thiol of GSH [18a]. The reactivity of the ethylene double bond was also postulated to play a crucial role in the inhibition of GST π [18b]. Therefore the reduction of the ethylene bond might severely reduce the reactivity of **EA** and its derivatives.

These results prompted us to consider modifications of the part 2 instead of the part 1 of the structure (Figure 2) including two series: series A (2-(4-substituted phenyl)ethanamine) and series B (4-(4-substituted phenyl)piperazine), and for this purpose **EA** derivatives **5-22** were prepared.

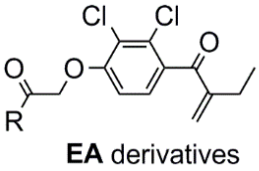
The anti-proliferative activities of **EA** and **5-17** are shown in Table 1. Thus, introduction of a 2-phenylethanamine chain (series A) affording the compound **5**, provided good anti-proliferative activity against both KB and HL60 cell lines with IC_{50} values of 0.6 µM and 1.3 µM, respectively. In addition, compound **5** showed low activities against EPC quiescent cells, with an IC_{50} of 4.2 µM, suggesting its specific action on rapidly proliferating cells, e.g. cancer cells. This encouraging result drove us to design other **EA** derivatives using amide functions. Thus, as shown in Scheme 2, we prepared the derivatives **6** and **7** bearing 4-hydroxy-2-phenylethanamine (series A) and 4-hydroxy-phenylpiperazine (series B) groups, respectively.

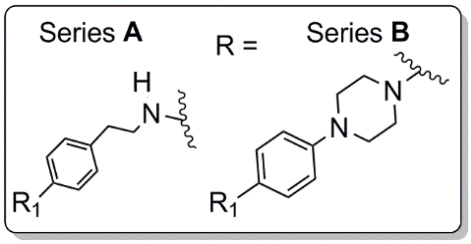
Both amide derivatives (**6** and **7**) displayed anti-proliferative activity against KB and HL60 cell lines with IC_{50}s of 0.4 µM and 0.7 µM, and 0.4 µM and 0.8 µM, respectively. In addition, both displayed a reduced activity against EPC cell lines, with IC_{50}s around 3-4 µM. Based on the promising results of these two active compounds, we decided to develop two different chemical series: 2-(4-substituted phenyl)ethanamine and 4-(4-substituted phenyl)piperazine. The simple substitution of the hydroxyl group of **6** and **7** by a methoxy moiety, afforded the compounds **8** and **9**, respectively. The compounds showed anti-proliferative activity against both KB and HL60 cell lines, with IC_{50}s of 0.8 µM and 1.3 µM, and 0.4 µM and 0.8 µM, respectively. Interestingly, the replacement of the hydroxyl group of **6** and **7** by a dimethyl phosphate moiety afforded the compounds **10** and **11** with IC_{50}s of 0.4 µM and 1.2 µM, and 0.17 µM and 0.8 µM against KB and HL60 cell lines, respectively. Its substitution by a diethyl phosphate moiety (compounds **12** and **13**), increased also the anti-proliferative activity *versus* **EA**, and maintain the anti-proliferative activity *versus* **10** and **11**, with IC_{50}s of 0.8 µM and 1.1 µM, and 0.3 µM and 0.5 µM against KB and HL60 cell lines, respectively. Importantly, the introduction of a cyclic phosphate (2-hydroxy-1,3,2-dioxaphosphinane 2-oxide) in position-4 on the phenyl groups of **6** and **7** instead of the hydroxyl group, afforded the **EA** derivatives **14** and **15**, displaying good anti-proliferative activities, and showing IC_{50}s of 0.5 µM and 1.2 µM, and 0.2 µM and 0.35 µM against KB and HL60 cell lines, respectively. From these derivatives, we then decided to prepare the derivatives **16** and **17** bearing a 3-chloro-phenyl group in position 4 of the 1,3,2-dioxaphosphorinane ring as cytochrome CYP3A4-selective prodrugs of the hydroxyl group of **6** and **7**. The **EA** derivatives **16** and **17** displayed similar anti-proliferative activities than **14** and **15**.

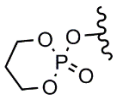
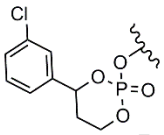
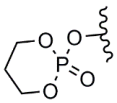
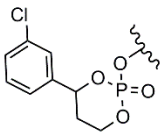
It is noteworthy that two of the most potent **EA** analogs within the series B (compounds **11** and **17**), exhibited very promising SI. Compound **11** was respectively 17- and 3.6-fold more potent on KB and HL60 than on EPC. Compound **17** was respectively 21- and 8.7-fold more potent on KB and HL60 compared to EPC.

The very interesting anticancer activities of the compound **8** (series A) prompted us to orientate our efforts towards the preparation of disubstituted **EA** derivatives **18**, **19** and **20** in order to evaluate the role of an additional group on the phenyl ring of **8** and **12**.

Table 1. Anti-proliferative activities of **EA** and compounds **5-17** (series A and B).





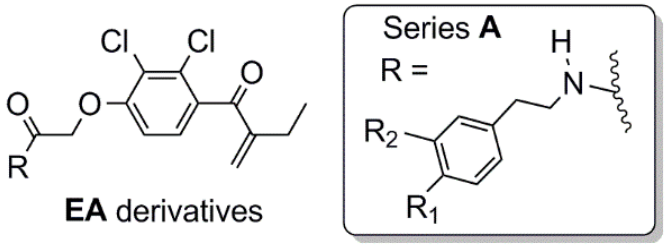
Series	Compounds	R_1	KB	IC_{50} (μM) HL60	EPC	Selectivity Index [a] KB/HL60	logP[b]
	EA		11±2	46±12	>100	>9 / >2	3.0
A	**5**	H	0.6±0.1	1.3±0.3	4.2±0.7	7 / 3	4.6
A	**6**	HO	0.4±0.05	0.7±0.1	3.6±0.1	9 / 5	4.3
A	**8**	MeO	0.8±0.1	1.3±0.1	3.7±0.1	4.6 / 2.8	4.5
A	**10**	$(MeO)_2P(O)$-O	0.4±0.1	1.2±0.1	0.7±0.2	1.75 / 0.58	4.7
A	**12**	$(EtO)_2P(O)$-O	0.8±0.1	1.1±0.1	3.2±0.2	4 / 2.9	5.4
A	**14**		0.5±0.1	1.2±0.2	3.4±0.1	6.8 / 2.8	4.6
A	**16**		0.7±0.2	0.9±0.1	2.2±0.2	3.1 / 2.4	7.0
B	**7**	HO	0.4±0.1	0.8±0.1	3.2±0.1	8 / 4	4.3
B	**9**	MeO	0.4±0.2	0.8±0.1	3.6±0.1	9 / 4.5	4.6
B	**11**	$(MeO)_2P(O)$-O	0.17±0.02	0.8±0.1	2.9±0.1	17 / 3.6	4.9
B	**13**	$(EtO)_2P(O)$-O	0.3±0.1	0.5±0.1	2.8±0.2	9.3 / 5.6	5.6
B	**15**		0.2±0.1	0.35±0.05	1.25±0.05	6.25 / 3.6	4.7
B	**17**		0.4±0.1	0.9±0.1	7.8±1.4	21 / 8.7	7.1

[a] Selectivity Index (SI): IC_{50}EPC/IC_{50}KB or HL60. [b] Calculated partition coefficient (logP), see text

As shown in Table 2, we observed no improvement in the anti-proliferative activity of **18** and **19** *versus* **8**. We then decided to introduce a methoxy group in position 3 of **12** due to its excellent anti-proliferative activity to afford the compound **20**. Also, we observed no improvement in the anti-proliferative activity of **20** *versus* **12**.

Then, to further investigate SAR within the series A, we turned our attention to the replacement of the NH group of **6** by either an oxygen or a *N*-methyl group affording compounds **21** and **22**, respectively. Both compounds **21** and **22** displayed good anti-proliferative activities against KB and HL60 (Table 3). Given that **22** displayed similar activities to **6**, the replacement of a secondary amide group with a tertiary amide group did not influence the cellular potency.

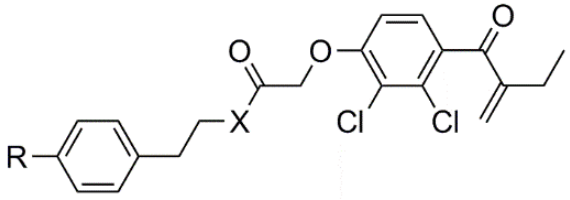
Table 2. Anti-proliferative activities of **EA**, **8**, **12** and **18-20**.



Series	Compounds	R_1	R_2	KB	IC_{50} (µM) HL60	EPC	Selectivity Index [a] KB / HL60	logP[b]
	EA			11±2	46±12	>100	>9 / >2	3.0
A	**8**	MeO		0.8±0.1	1.3±0.1	3.7±0.1	4.6 / 2.8	4.5
A	**12**	$(EtO)_2P(O)$-O		0.8±0.1	1.1±0.1	3.2±0.2	4 / 2.9	5.4
A	**18**	HO	MeO	0.8±0.1	1.6±0.1	4.5±0.3	5.6 / 2.8	4.6
A	**19**	MeO	MeO	0.5±0.1	0.8±0.1	3.4±0.1	6.8 / 4.2	4.4
A	**20**	$O\text{-}P(O)(OEt)_2$	OMe	0.7±0.1	0.8±0.1	2.3±0.1	3.2 / 2.8	5.1

[a] Selectivity Index (SI): IC_{50}EPC/IC_{50}KB or HL60.[b] Calculated partition coefficient (logP), see text

Table 3. Anti-proliferative activities of **EA**, **6**, **21** and **22**.



Compounds	R	X	KB	IC_{50} (µM) HL60	EPC	Selectivity Index[a] KB / HL60	logP[b]
EA			11±2	46±12	>100	>9 / >2	3.0
6	HO	NH	0.4±0.05	0.7±0.1	3.6±0.1	9 / 5	4.3
21	OH	O	0.9±0.1	1.4±0.1	3.7±0.1	4 / 2.65	4.9
22	OMe	N-Me	0.7±0.05	1.3±0.1	4±0.3	5.7 / 3	4.8

[a] Selectivity Index (SI):IC_{50}EPC/IC_{50}KB or HL60. [b] Calculated partition coefficient (logP), see text

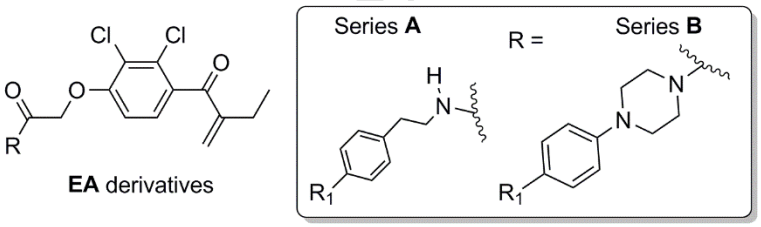
Finally, in order to fully confirm the essential role of the α,β unsaturated carbonyl moiety of the derivatives **6** (series A), **7** and **9** (series B), we focused our attention on the synthesis of compounds **23** and **24** (series A), and compounds **25** and **26** (series B) (Scheme 6). As previously observed for the compounds **3** and **4**, the selective reduction of the α,β unsaturated carbonyl moiety totally abolished the anti-proliferative activity of **6**, **7** and **9** with IC_{50}s >100 μM against both KB, HL60 or EPC.

Then, we attempted to correlate the anti-proliferative activities of **EA** derivatives with a classical physicochemical parameter such as calculated membrane-water partition coefficient (logP). It is well known that lipophilicity, which associates both biological and physicochemical properties, is one of the most important physical properties of a drug conditioning its absorption, distribution, potency and elimination [19,20]. For each compound prepared (Tables 2-4) we calculated the corresponding logP using Marvin application (ChemAxon). No relationship can be reported between logP and IC_{50} but readily reveals that a potent anti-proliferative activity was observed when logP is higher than 4 (ranging 4.3-7.1) *versus* 3 for **EA**. It is important to note that the logP must be not too high in order to develop drug-like compounds (*e.g.* solubility issue). Additionally, no correlation has been noticed between IC_{50}s and either pKa, MW, the number of hydrogen acceptors, or the number of hydrogen donors using Marvin application (ChemAxon).

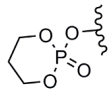
Based on the anti-proliferative activity and selectivity index, compounds **11** and **17** are the most interesting derivatives. They showed a SI of **17** and **21**, respectively (KB *vs* EPC) and displayed similar but higher potency against KB in comparison with HL60. The most promising is the compound **11** due to its lower logP (4.9 *vs* 7.1 for **17**) and lower molecular weight (529 *vs* 651).

For further investigations, we decided to explore the anti-proliferative activity of a series of chemical diverse **EA** derivatives (series A and B) against a panel of cancer cell lines representative of diverse tissues/organ tumors: human breast cancer cell line (MCF7), human colon carcinoma cell line (HCT116), human prostate cancer cell line (PC3), ovarian carcinoma cells (SK-OV3 and OVCAR8, Table 4).

Table 4. Anti-proliferative activity of **EA** and derivatives upon various cancer human cells.



Series	Compounds	R_1	IC_{50} μM)[a] MCF7	HCT116	PC3	SK-OV3	OVCAR8
	EA		41±9	75±0	75±0	>100	72±3
A	**6**	HO	1.8±0.2	1.7±0.6	1.0±0.5	4.5±1.8	1.5±0.3
A	**10**	$(MeO)_2P(O)$-O	0.9±0.1	1.5±0.6	0.8±0.2	2.8±0.2	1.5±0.2
B	**7**	HO	0.8±0.1	2.0±0.7	3.9±3.1	2.3±1.2	0.8±0.3
B	**11**	$(MeO)_2P(O)$-O	0.5±0.1	0.8±0.5	1.1±0.1	2.2±1.3	1.4±0.2

B	**15**		0.9±0.4	1.0±0.6	0.5±0.1	3.5±0.5	1.9±0.6

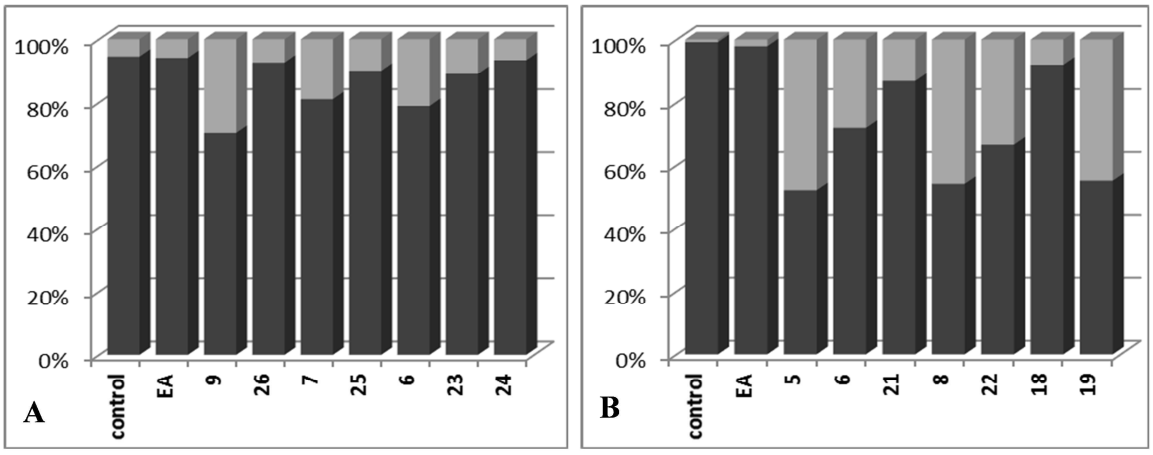
EA retained a poor anti-proliferative activity against the five cell lines tested with IC_{50} above 50 µM, featuring its general lack of activity, while the **EA** derivatives **6**, **7**, **10**, **11** and **15** displayed moderate to good anti-proliferative activities with IC_{50}s ranking between 4.5 µM and 0.5 µM. In the series A, the derivative with a trimethoxy phosphate group linked to the 4-hydroxy-2-phenylethanamine moiety (**10**) was a potent anti-proliferative compound with IC_{50} slightly better than **6** having only the 4-hydroxy-2-phenylethanamine group (except against OVCAR8 cell line). This suggests that the introduction of a trimethoxyphosphate group moderately increased the anti-proliferative activity of **EA** derivatives. The replacement of the 4-hydroxy-2-phenylethanamine group by the 4-hydroxy-phenylpiperazine group (**7** *versus* **6**) had low significant effect except against PC3 cell lines for which **6** is about 4-fold better than **7**. Within the series B, the addition of the trimethoxyphosphate unit instead of the hydroxyl group on the phenyl ring (**11** *versus* **7**) or the introduction of a cyclic phosphate (oxo 2, 1,3,2-dioxaphosphorinane ring) also on the phenyl group (**15** *versus* **7**) moderately increases the potency except for the SK-OV3 and OVCAR8 cell lines.

All together these data suggested that the nature, hydrophobicity and size of the terminal group in position 4 of the phenyl unit linked to the **EA** molecule and the series (A and B) had no noticeable importance for the anti-proliferative activity of **EA** derivatives. It is noteworthy that the two phosphate derivatives **10** and **11** and the dioxaphosphorinane **15** showed good homogeneity related to their IC_{50}s and higher activity towards MCF7, HCT116 and PC3 cell lines than towards the ovarian cancer cell lines SK-OV3 and OVCAR8. A different sensibility to cisplatin has been reported in these two ovarian cell lines, SK-OV3 been considered as resistant compared to OVCAR8: a similar behavior can be observed with **EA** and its derivatives [21].

The next step was the determination of the mechanism of action of **EA** derivatives and was intended to confirm the modulation of anti-proliferative capacity by structural modifications. Evaluations regarding vital cellular processes have been tested essentially in HL60 cells for relevant comparison with a selection of 13 chemically diverse **EA** derivatives selected in accordance with their structural design and bearing modification either in the part A alleviating activity (**23**, **24**, **25** and **26**) or in the part B within the two series A and B: amide-monosubstituted (**5**, **6**, **7**, **8** and **9**), ester-monosubstituted (**21**), *N*-methyl amide-monosubstituted (**22**), and disubstituted (**18** and **19**) showing IC_{50}s against KB and HL60 between 0.4 µM and 1.6 µM.

To ensure the cytotoxicity of **EA** and its derivatives, the viability of HL60 cells was investigated by Fluorescence Activated Cell Sorter (FACS) after a 24h exposure to a selection of compounds chosen for their structure-activity determined previously. The viacount® reagent used in these experiments is composed of two DNA binding dyes, one permeant to viable cells and one impermeant allowing the discrimination between the number of viable and the total number of cells remaining after chemical exposure. The IC_{50} of **EA** (measured with the NADH reagent) was comparable at 24h and 72h in HL60 cells resulting from its poor anti-proliferative activity. Although lower than at 72h, anti-proliferative activity of **EA**

derivatives was higher than those of **EA** at 24h (Figure 4). Taking this into account, concentrations used for testing were 50 µM for **EA** and 7 µM for derivatives.



HL60	**EA**	**5**	**6**	**21**	**8**	**22**	**18**	**19**
IC_{50} (µM)	38±1	1.6±0.1	1.7±0.1	2.9±0.4	2.4±0.3	2.3±0.1	2.6±0.1	1.4±0.1

Figure 4. Anti-proliferative activity of **EA** (50 µM) and derivatives (7 µM) upon HL60 cells after a 24h exposure and cytotoxic activity measured by FACS. Results are expressed as the percentage of viable cells (in black) and dead cells (in light grey). IC_{50} were determined with the MTS test after a 24h exposure of HL60 cells.

The consequences of modification of the part 1 of the native **EA** molecule were not tested due to the very low basal activity of the **EA** molecule. First, modifications on the α,β unsaturated carbonyl moiety of **EA** derivatives were examined (Figure 2), the reduction of the ethylene bond of the 4-hydroxy-phenylpiperazine (**25**, **26** *vs* **7**) and 4-hydroxy-2-phenylethanamine (**23** *vs* **6**) linked chain resulted in a noticeable increase in the cellular viability to reach the value obtained with the native **EA** molecule. Reduction of the carbonyl bond (**24** *vs* **6**) also resulted in the loss of toxicity. These data are in line with those collected by measuring the reduced nicotinamide adenine dinucleotide (NADH) content of cells after 72h and therefore confirmed the critical role of the α,β unsaturated carbonyl moiety chain in the cell death promoted by **EA** derivatives.

As expected, replacement of the amido by an ester linkage (**21** *vs* **6**) severely impaired the toxicity of the derivative, in relation with the supposed hydrolysis of the ester bond (Table 3). The combined presence of a hydroxy and a methoxy group on the phenyl ring of the phenylethanamine chain (**18**) also augmented the IC_{50} at 24h and the viability of HL60 cells compared to compounds having a phenyl (**5**), a phenyl ring with a single hydroxy or methoxy group (**6** and **8**), or a dimethoxy (**19**) leading to potent cytotoxic molecules. The presence of a -N(CH_3)-CO- linkage instead of a -N(H)-CO- one (**22** *vs* **8**) had no effect.

Another marker to estimate the viability of cells consists in the determination of the cellular reduced glutathione content, which is an essential cellular component protecting from

damages caused by reactive oxygen species and electrophilic compounds [22]. Reduced glutathione content was only moderately decreased after a 24h exposure to 50 µM **EA**, whereas potent anti-proliferative derivatives at concentration 7 µM elicited a higher reduction (Figure 5). Once more the replacement of the amido linkage by an ester linkage strongly prevented the reduction of glutathione content (**21** *vs* **6**), as well as the presence of the 3-methoxy-4-hydroxy-2-phenylethanamine chain (**18**), compared to single substitution compounds (**6** and **8**) or to the dimethoxy 2-phenylethanamine derivative (**19**), potent inducers of GSH depletion.

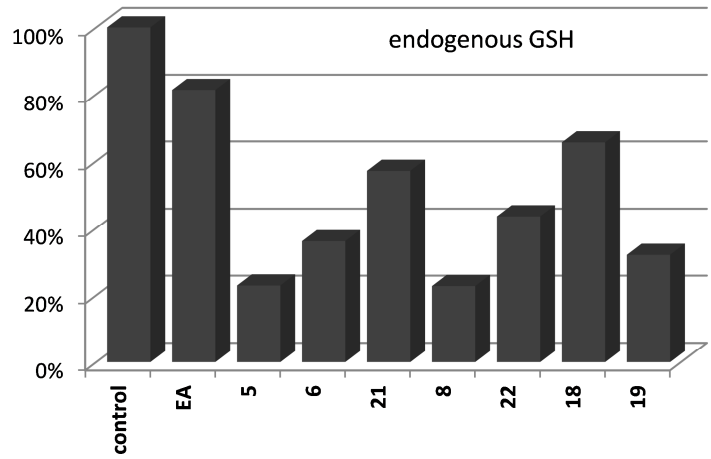


Figure 5. GSH content of HL60 cells exposed for 24h to **EA** (50 µM) and derivatives (7 µM). Results are expressed as a percentage of the GSH content in cells treated with DMSO alone.

Actually the cell viability estimated by FACS and the endogenous glutathione content were highly correlated (Figure 6), viable cells having a high glutathione content. This suggests that after a 24h exposure to **EA** derivatives, the cell death is already proceeding and results in the diminution of the reduced glutathione content, a reduction of the cellular NADH content, and finally the decrease of cell viability.

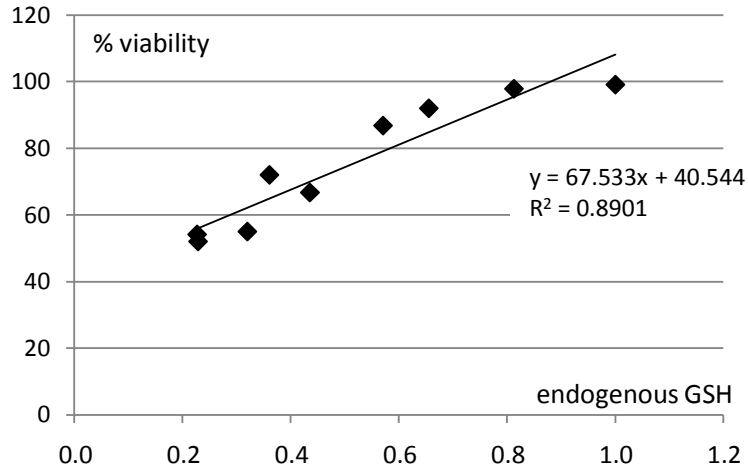


Figure 6. Relationship between cell viability and GSH content of HL60 cells exposed to **EA** (50 µM) and derivatives (7 µM) for 24h. Results are expressed as a percentage of values in cells treated with DMSO.

To ascertain the mode of action of **EA** derivatives, we intended to evaluate the different biological pathways leading to cell death. A first attempt was to explore a possible necrotic process [23] induced by **EA** derivatives in a set of experiments performed on HL60 cells. Necrosis results from the disruption of the plasma membrane and leads to the release of the cytosolic content. Therefore, necrosis can be estimated by measuring the activity of lactate dehydrogenase (LDH), released into the culture medium during treatment. Menadione, which is known to induce necrosis in various cell lines, was used as a positive control. At 50 µM, menadione elicited an increase of the LDH activity released in the culture medium up to 60% after a 48h exposure (data not shown) and a reduction of the total cell number. Exposure of HL60 cells to 50 µM **EA** had only a marginal effect on the LDH ratio after 24 and 48h, confirming the poor intrinsic cytotoxicity of this molecule (Figure 7, upper panel). After a 24h exposure to **EA** derivatives, the release of LDH was limited in HL60 cells and only a 48h exposure to a few **EA** derivatives resulted in a significant but moderate release of LDH: the dimethoxy 2-phenylethanamine linked **EA** (**19**) was the more active followed by the 4-methoxy-2-phenylethanamine *N*-methyl amido **EA** (**22**) and the 4-methoxy-2-phenylethanamine **EA** (**8**). A similar moderate effect was obtained within KB cells (Figure 7 middle panel) and also in HCT116 and MRC5 cells (data not shown).

Modifications on the α,β unsaturated carbonyl moiety of **EA** derivatives were also examined (Figure 7 lower panel). The reduction of the ethylene double bond of the 4-hydroxy-phenylpiperazine (**26** and **25** *vs* **7**) or 4-hydroxy-2-phenylethanamine (**23** *vs* **6**) linked chain or reduction of the carbonyl of the 4-hydroxy-2-phenylethanamine linked chain (**24**) did not modified substantially the LDH release. These results demonstrate that the necrotic process is only marginally involved in the biological effect of **EA** derivatives.

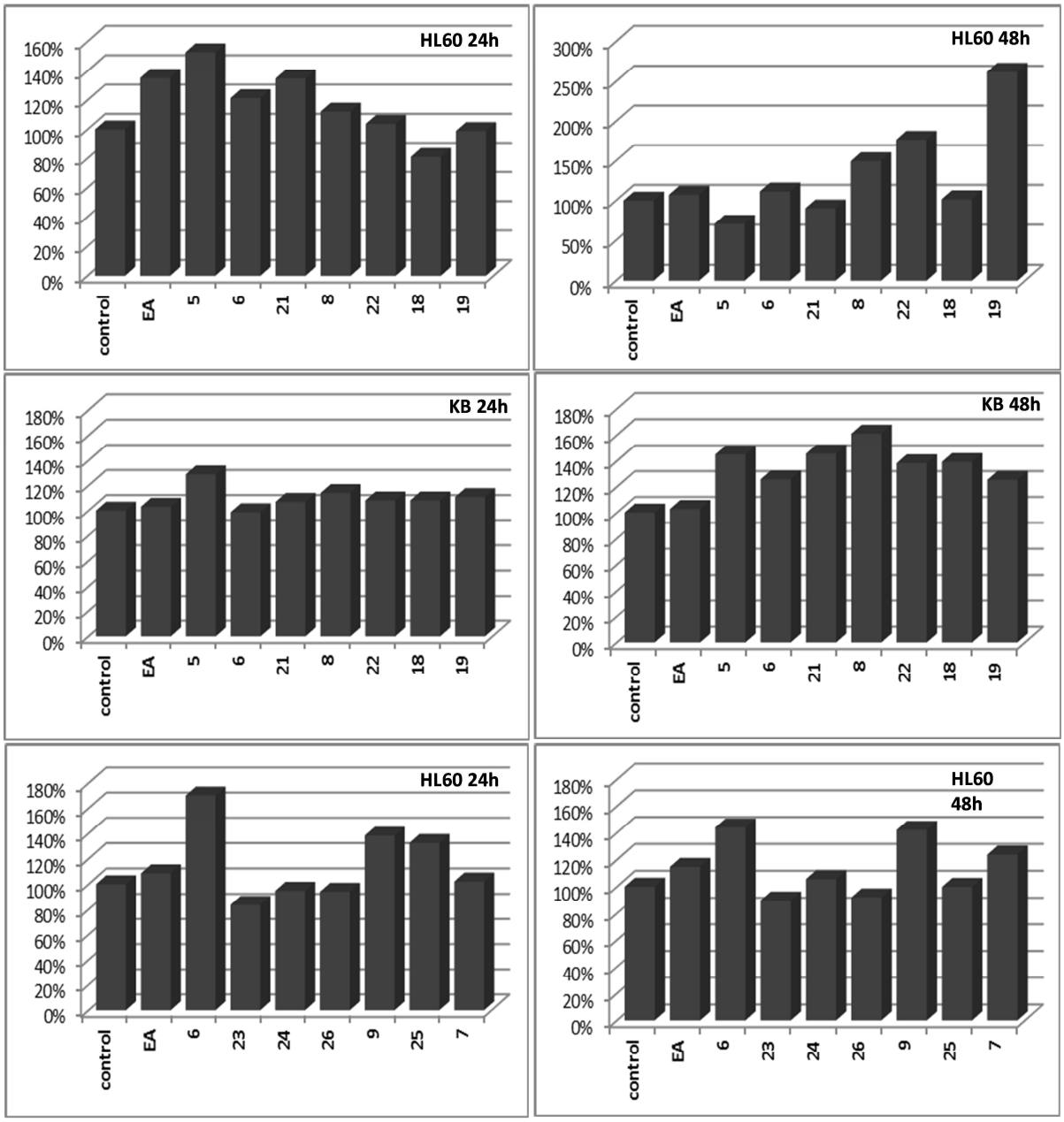


Figure 7. LDH release from cells treated for 24 and 48h with **EA** (50 µM) and derivatives (7 µM). Results are the amount of LDH released in the culture medium from KB and HL60 cells treated for 24 or 48h with **EA** and derivatives and expressed as a percentage of LDH released by cells receiving DMSO alone.

Several markers can be utilized to characterize apoptosis which is a common pathway used by anti-proliferative drugs to promote cell death [24]. Flow cytometry analysis (FACS) is a reliable and fast method to demonstrate the entry of cells into the apoptotic process. An early event in the apoptotic process is the externalization of phosphatidyl-serine from the inner to the outer cell membrane, then accessible to annexin V. Later the plasma membrane is disrupted during the late apoptosis/necrosis stage and cells can be classified as intact, in early apoptosis or in late apoptosis or necrosis. To further analyze the biological effect of **EA** derivatives, annexin-V-7 AAD staining was carried out and assessed by flow cytometry. Therefore, HL60 cells were treated for 24h with **EA** and four of its derivatives selected for their activity. Results are shown in Table 5. Control cells were treated with vehicle only. **EA**

at low concentration had no effect after 24h, but induced the cell entry in early apoptosis at high concentration.

Table 5. FACS analysis of cell death induced by **EA** and derivatives after 24h exposure.

Compound	dead cells	necrosis/late apoptosis	intact cells	early apoptosis
Control	0.1	0.6	91.6	7.7
EA (10 µM)	0.1	0.9	87.8	11.2
EA (50 µM)	0	0.8	57.9	41.3
5 (5 µM)	0.3	21.9	12.8	65
6 (5 µM)	0.3	16.0	33.7	50
21 (5 µM)	0.2	10.0	47.3	42.5

EA derivatives promoted early apoptosis at a lower concentration (5 µM instead of 10 or 50 µM) and also late apoptosis/necrosis: as expected from former data, the replacement of the amido linkage by the ester linkage (**21** *vs* **6**) reduced the entry of cells in the apoptotic process, especially in the late apoptosis/necrosis stage. The nature of the substituent on the NH amino linkage had no effect since all derivatives displayed comparable capacity to activate apoptosis (data not shown). Similar data were observed after a 48h exposure (data not shown) indicating that the activation of the apoptotic pathway was a rapid answer to chemical exposure. Therefore it is conceivable to conclude that **EA** derivatives induced cell death through the activation of the apoptotic pathway.

Caspases (cysteine-aspartic acid proteases) are activated during apoptosis in many cells and are known to play a vital role in both initiation and execution of apoptosis. It was reported that caspase-3 is the terminal effector in the apoptotic cascade and is a major contributor to cellular DNA fragmentation. To go further in the elucidation of the apoptotic pathway stimulated by **EA** derivatives, the catalytic activity of caspase-3 was assayed in HL60 cells treated for 24h with **EA** (50 µM) and the selected **EA** derivatives (5 µM). Doxorubicin (50 nM) was used as a positive control. At low concentration, **EA** failed to activate caspase activity, in line with data obtained by FACS, but unexpectedly had also no effect at high concentration (Figure 8). Consistent with the entry in early apoptosis observed above, **EA** derivatives activated to a great extent caspase 3 after a 24h exposure: 2-phenylethanamine (**5**) and 4-hydroxy-2-phenylethanamine (**6**) linked derivatives elicited comparable striking activation, whereas the ester linked analog (**21**) was much less active.

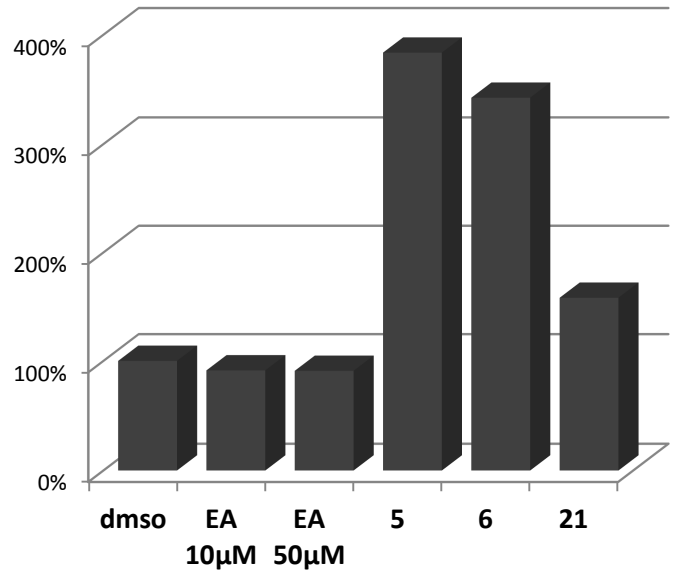


Figure 8. Activation of caspase 3/7 activity in HL60 cells by **EA** (50 µM) and derivatives (5 µM) after 24 h exposure.

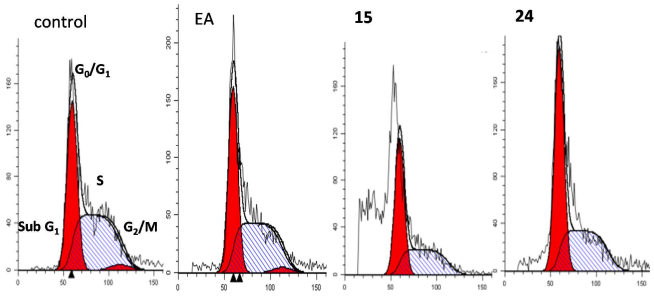
The Jun-JNK signaling pathway is a mechanism proposed to explain caspase activation. JNK is a pro-apoptotic kinase [25a] associated to GST π in the cell cytosol then impairing the kinase activity [25b]. The exposure to GST π inhibitors prevents the repression of JNK activity and impacts apoptosis [25b,c]. Previously, **EA** at high doses was found to synergize the arsenic oxide action in HL60 cells, through the activation of JNK and caspase 3 [26]. Therefore it is conceivable that the active **EA** derivatives might be potential inhibitors of GST π, produce an activation of JNK to finally stimulate apoptosis.

Another mechanism involves the Wnt/β-catenin pathway. Overexpression of β-catenin induces apoptosis in hematopoietic stem/progenitor cells and NIH 3T3 fibroblasts via the elevation of caspase 3 [27a,b]. **EA** and **EA** amine derivatives have been reported as Wnt inhibitors in a cell based Wnt reporter assay and selectively suppressed chronic lymphocytic leukemia (CLL) cells survival although no strict relationship was observable between CLL inhibition growth and Wnt inhibition [12c, 27c]. The inhibition of Wnt by active **EA** derivatives described in this study might explain their antiproliferative capacity.

It has been assumed that anti-proliferative drugs triggering apoptosis and cell death, ultimately promoted DNA fragmentation associated or not with prior cell cycle arrest in either G_1, S or G_2/M phases. To ascertain whether or not **EA** and its derivatives are capable to interact with the cell cycle, the impact of 2.5 µM **EA** derivatives was investigated in HL60 cells treated for 24 and 48h. For that purpose, we selected the **EA** derivatives **6**, **7**, **15** derivatives having high anti-proliferative activity, as well as two inactive derivatives **23**, **24**. Cells were stained with propidium iodide (PI), and analyzed by FACS: a majority of control cells contained 2n chromosomes (phase G_0/G_1) or participated in an active DNA synthesis (phase S), with some cells already engaged in mitosis (phase G_2/M). In apoptotic cells the DNA was partly fragmented (phase sub G_1) (Table 6). After a 24h exposure to either derivative, no modification of the proportion of cells containing 2n, intermediate and 4n chromosomes was noticeable. After 48h, 2.5 µM **EA** did not alter the partition of cells between the cell cycle phases, whereas active anti-proliferative **EA** derivatives **6**, **7** and **15** rose the proportion of apoptotic cells with a reduction of cells in S phase and an almost complete disappearance of G_2/M phase. As anticipated, the two inactive **EA** derivatives **23** and **24** did not raise the proportion of apoptotic cells, but unexpectedly reduced the number of

cells in phases S and G_2/M and augmented the G_0/G_1 phase. It could be concluded that **EA** had no effect on the control of cell cycle, while its active derivatives prevented the DNA synthesis and mitosis to favor resting cells before to promote apoptosis and DNA fragmentation.

Table 6. Cell cycle analysis by FACS of HL60 cells treated for 24 and 48h with **EA** and derivatives (2.5 µM)



Compounds	G0/G1	S Phase	G2/M	SubG1
Control 24h	43.5	48.9	5.1	2.6
EA	45.4	43.6	8.3	2.7
6	43.2	47.3	6.2	3.3
7	41.7	49.3	6.1	2.9
15	40.7	47.1	9.6	2.6
23	42.1	46.7	7.9	3.3
24	42.3	50.5	5.2	2.0
Control 48h	43.8	52.6	2.2	1.4
EA	41.5	54.1	1.3	3.1
6	44.3	45.8	0.1	9.9
7	49.3	33.8	0.4	16.5
15	41.7	30.7	0.0	27.6
23	51.7	41.9	2.2	4.2
24	51.9	41.5	0.6	6.1

4. Conclusions

The starting point of this original study is the diuretic drug ethacrynic acid which displays by itself a modest anti-proliferative activity at high doses (>60 µM) [10a] and is believed to have no direct effect on cell death but acts as inhibitor of π class glutathione *S*-transferase, in line with its role of adjuvant in chemotherapy. This low intrinsic anti-proliferative activity could be markedly enhanced through the introduction of an additional lateral chain affording original **EA** derivatives. Clearly, our data feature several points:

- the presence of the α-β double bond of **EA** is essential for its cytotoxic activity, any alteration of this residue resulting in a total loss of activity.
- addition of a lateral chain raising the logP results in a drastic increase of the anti-proliferative capacity, reduces the intracellular glutathione content and finally the cell viability.
- the replacement of the stable amido bond by a cleavable ester bond generates the release of native **EA** and significantly lowers the anti-proliferative activity of derivatives.

- when the α-β double bond of active **EA** derivatives is modified, the anti-proliferative activity is totally lost confirming the crucial involvement of the α-β double bond in the cell death process.

Therefore, the anti-proliferative activity of **EA** derivatives prepared during this study is the combinational result of two elements: the basic toxicity due to the α-β double bond and its potentiation by the addition of a hydrophobic lateral chain. Thus, it becomes possible to modulate the poor anti-proliferative capacity of **EA** by introduction of relevant lateral chain leading to the activation of the apoptotic cascade.

In summary, this study points out the potential of new **EA** derivatives as novel efficient anticancer agents, with enhanced potency relative to **EA** itself. Basically and interestingly, the active **EA** derivatives prepared in this study induce primarily the apoptosis process without activation of necrosis, through the activation of the caspase cascade without alteration of the cell division, while in marked contrast inactive **EA** derivatives promote cell resting. This mechanism of action of the **EA** derivatives reported in this study is not single - which potentially will avoid tumor resistances - than the unique mechanism of action established for **EA**, which is an inhibitor of π class glutathione *S*-transferase.

Clearly our study demonstrates the optimization of **EA** which shows low anti-proliferative activities (IC_{50} > 50 µM) with unclear/several mechanism(s) and affords potent, original and simple **EA** derivatives (IC_{50} 0.2-1.6 µM) through apoptotic process.

5. Experimental Section

Syntheses were carried out using standard high vacuum and dry-argon techniques. All chemicals were purchased from Across, Aldrich, Fluka, and used without further purification. The solvents were freshly dried and distilled according to standard procedures prior to use. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with Bruker AV300, DPX300, AV400, spectrometers. All ^{13}C NMR and ^{31}P NMR spectra were generally recorded decoupled {^{1}H}. The signal of the non-deuterated solvent served as internal standard. The following abbreviations have been used for indicating the multiplicity of signals: s (singlet), d (doublet), q (quadruplet), h (heptuplet), m (multiplet). Fourier transformed infrared (FTIR) spectra were obtained with a Perkin–Elmer Spectrum 100 FT-IR spectrometer on neat samples (ATR FT-IR) or in solutions. Mass spectrometry was carried out with a Thermo Fisher DS QII (DCI/NH_3 or DCI/CH_4). Compounds **3** [12c], **4** [28] as well as compounds **6** and **7** [10] were prepared following the previously reported procedure. The methyl ester of **EA**, which is the intermediate for the preparation of compound **4**, was prepared according to reference [29]. The chloro-2-oxo-2-dioxophosphorinase used for the synthesis of **14** and **15** was prepared according to reference [13]. The 2-chloro-4-(3-chlorophenyl)-1,3,2-dioxaphosphorinane 2-oxide used for the preparation of **16** and **17** was prepared according to reference [17], 2-(2,3-dichloro-4-(2-methylbutanoyl)phenoxy)acetic acid (**3**) according to reference [12c] and 2-(2,3-dichloro-4-(1-hydroxy-2-methylenebutyl)phenoxy)acetic acid (**4**) according to reference [30].

General Procedure: Preparation of **5**, **8**, **9**, **19** and **22**. To a mixture of EDCI (1.2 equiv), catalytic amount of DMAP and ethacrynic acid (1 equiv) in dry DMF (5 mL), amine (2-phenylethanamine, 2-(4-methoxyphenyl)ethanamine, 2-(4-methoxyphenyl)-N-methylethanamine, 4-(2-aminoethyl)-2-methoxyphenol, 2-(3,4-dimethoxyphenyl)ethanamine) and 1-(4-methoxyphenyl)piperazine (1 equiv) was added at 0°C. The reaction mixture was stirred overnight at room temperature. Then, ethyl acetate (100 mL) was added and the organic layer was washed with water (2 × 50 mL) and brine (3 × 50 mL), dried over

anhydrous $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by flash chromatography.

2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-*N*-phenethylacetamide (5). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:8 (v/v) to THF, as eluents, to furnish **5** as a white solid (155 mg, yield of 58%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.31-7.38 (m, 2H, Har), 7.24-7.30 (m, 3H, Har), 7.21 (d, *J* = 8.5 Hz, 1H, Har), 6.89 (d, *J* = 8.5 Hz, 1H, Har), 6.74 (s, 1H), 6.00 (s, 1H), 5.61 (s, 1H), 4.57 (s, 2H), 3.67 (q, *J* = 6.9 Hz, 2H), 2.91 (t, *J* = 6.9 Hz, 2H), 2.49 (q, *J* = 7.4 Hz, 2H), 1.18 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 195.3 (C, C=O), 166.3 (C, C(=O)N), 154.6 (C, Car), 150.2 (C, Car), 138.6 (C, Car), 133.9 (C, Car), 131.0 (C, Car), 128.7 (2CH, Car), 128.6 (2CH, Car), 128.7 (CH_2, C=CH_2), 127.2 (CH, Car), 126.6 (C, Car), 122,7 (C, Car), 110.9 (CH, Car), 68.2 (CH_2, OCH_2), 39.9 (CH_2), 35.4 (CH_2), 23.4 (CH_2), 12.2 (CH_3). IR (neat): ν = 3404 (NH), 1661(C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{21}H_{21}Cl_2NO_3$: 406.0977, found: 406.0972.

2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-*N*-(4-methoxyphenethyl)acetamide (8). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 3:7 (v/v) to THF, as eluents, to furnish **8** as a white solid (181 mg, yield of 63%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.18 (d, *J* = 8.6 Hz, 1H, Har), 7.13 (d, *J* = 8.7 Hz, 2H, Har), 6.87 (d, *J* = 8.7 Hz, 2H, Har), 6.83 (d, *J* = 8.6 Hz, 1H, Har), 5.97 (s, 1H), 6.75 (s, 1H, NH), 5.59 (s, 1H), 4.55 (s, 2H), 3.82 (s, 3H, OCH_3), 3.65 (q, *J* = 6.6 Hz, 2H), 2.84 (t, *J* = 6.6 Hz, 2H), 2.49 (q, *J* = 7.4 Hz, 2H), 1.17 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 195.5 (C, C(=O)), 166.5 (C, C(=O)N), 158.4 (C, Car), 154.4 (C, Car), 150.2 (C, Car), 134.1 (C, Car), 131.4 (C, Car), 130.1 (C, Car), 129.7 (2CH, Car), 128.7 (CH_2, C=CH_2), 127.2 (CH, Car), 122.9 (C, Car),114.2 (2CH, Car), 110.7 (CH, Car), 68.1 (CH_2), 55.3 (OCH_3), 40.2 (CH_2), 34.7 (CH_2), 23.4 (CH_2), 12.4 (CH_3). IR (neat): ν = 3404 (NH), 1661(C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{22}H_{23}Cl_2NO_4$: 436.1082, found: 436.1094.

1-(2,3-dichloro-4-(2-(4-(4-methoxyphenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)-2-methylenebutan-1-one (9). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 8:2 (v/v) as eluent, to furnish **9** as a white solid (180 mg, yield of 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.15 (d, *J* = 8.5 Hz, 1H, Har), 6.99 (d, *J* = 8.5 Hz, 1H, Har), 6.93-6.82 (m, 4H, Har), 5.93 (s, 1H), 5.59 (s, 1H), 4.86 (s, 2H), 3.83-3.73 (m, 7H), 3.08 (t, *J* = 5.0 Hz, 2H), 3.03 (t, *J* = 5.0 Hz, 2H), 2.46 (q, *J* = 7.4 Hz, 2H), 1.14 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.9 (C, C(=O)), 165.3 (C, C(=O)N), 155.4 (C, Car), 154.7 (C, Car), 150.3 (C, Car), 145.2 (C, Car), 133.9 (C, Car), 131.7 (C, Car), 128.8 (CH_2, C=CH_2), 127.3 (2CH, CHar), 123.0 (C, Car), 119.3 (CH, CHar), 114.7 (2CH, CHar), 110.9 (CH, CHar), 68.9 (CH_2), 55.7 (OCH_3), 51.6 (CH_2), 51.0 (CH_2), 45.8 (CH_2), 42.6 (CH_2), 23.7 (CH_2), 12.6 (CH_3). IR (neat): ν = 1661 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{24}H_{26}Cl_2N_2O_4$ 477.1348, found 477.1324. Elemental analysis calculated (%) for $C_{24}H_{26}Cl_2N_2O_4$: C 60.38, H 5.49, N 5.85, found: C 60.42, H 5.24, N 5.69.

2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-*N*-(3,4-dimethoxyphenethyl)acetamide (19). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:8 (v/v) to THF, as eluents, to furnish **19** as a white solid (138 mg, yield of 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.21 (d, *J* = 8.6 Hz, 1H, Har), 6.90 (d, *J* = 8.6 Hz, 1H, Har), 6.69-6.91 (m, 4H, Har), 6.00 (s, 1H), 5.60 (s,1H), 4.56 (s, 2H), 3.84 (s, 6H), 3.65 (q, *J* = 6.6 Hz, 2H), 2.84 (t, *J* = 6.9 Hz, 2H), 2.49 (q, *J* = 7.6 Hz, 2H), 1.17 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 195.3 (C, C(=O)), 166.3 (C, C(=O)N), 154.6 (CH, Car), 150.2 (CH, Car), 149.3 (C, Car), 148.0 (C, Car), 133.9 (C, Car), 131.0 (C, Car),130.9 (C,

Car), 128.59 (CH, Car), 127.2 (CH, Car), 122.7 (C, Car), 120.7 (CH, Car), 112.1 (CH, Car), 111.7 (CH, Car), 110.9 (CH, Car), 68.3 (CH_2), 55.8 (OCH_3), 55.7 (OCH_3), 40.1 (CH_2), 35.0 (CH_2), 23.4 (CH_2), 12.2 (CH_3). IR (neat): ν = 3404 (NH), 1661(C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{23}H_{25}Cl_2NO_5$: 466.1188, found: 466.1188.

2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-*N*-(4-methoxyphenethyl)-*N*-methylacetamide (22). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:8 (v/v) to THF, as eluents, to furnish **22** as a white foam (184 mg, yield of 58%).Two conformations (50% / 50%) were observed at room temperature. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.09-7.16 (m, 3H, Har), 7.03-7.09 (m, 3H, Har), 6.84-6.91 (m, 3H, Har), 6.05 (d, *J* = 1.6 Hz, 1H, Har), 5.95 (s, 1H), 5.94 (s, 1H), 5.63 (s, 5H), 5.61 (s, 1H), 4.80 (s, 1H), 4.44 (s, 1H), 3.80 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.61 (t, *J* = 6.8 Hz, 4H), 3.03 (s, 3H, NCH_3), 2.98 (s, 3H, NCH_3), 2.86 (t, *J* = 6.6 Hz, 2H), 2.80 (t, *J* = 7.2 Hz, 2H), 2.46-2.57 (m, 4H), 1.17 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (126 MHz, C_6D_6) δ = 195.92 (d, C, C(=O)), 195.86 (d, C, C(=O)), 166.59 (C, C(=O)N),166.25 (C, C(=O)N), 158.34 (C, Car), 157.79 (C, Car), 155.30 (C, Car), 155.22 (C, Car), 149.53 (C, Car), 132.88 (C, Car), 132.62 (C, Car), 131.15 (C, Car), 130.84 (C, Car), 130.45 (CH, Car), 130.43 (CH, Car), 130.36 (CH_2, C=CH_2), 130.26 (CH_2, C=CH_2), 130.04 (CH, Car), 129.81(C, Car), 127.17 (CH, Car), 127.03 (CH, Car), 122.42 (C, Car), 122.04 (C, Car), 114.28 (CH, Car), 113.74 (CH, Car), 110.32 (CH, Car), 110.21 (CH, Car), 67.42 (CH_2), 66.77 (CH_2), 55.46 (OCH_3), 55.44 (OCH_3), 50.76 (CH_2), 50.41 (CH_2), 35.49 (NCH_3), 33.45 (NCH_3), 32.92 (CH_2), 32.53 (CH_2), 23.27 (CH_2), 23.24 (CH_2), 12.4 ($2CH_3$). IR (neat): ν = 1658 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{22}H_{23}Cl_2NO_4$: 450.1239, found: 450.1235.

General Procedure: Preparation of **10**, **11**, **12**, **13**, **14**, **15**, **16**, **17** and **20**. To a mixture of the corresponding phenol (1 equiv) and triethylamine (1.1 equiv) in dry DCM at 0 °C, chlorophosphate was added dropwise (1 equiv). The reaction mixture was allowed to warm at room temperature, then stirred at that temperature overnight. DCM (20 mL) was added and the organic layer was washed with water (10 mL) and brine (10 mL), dried over anhydrous $MgSO_4$ and then concentrated under reduced pressure. The crude product was purified by flash chromatography.

4-(2-(2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetamido)ethyl)phenyl dimethyl phosphate (10). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 8:2 (v/v) as eluent, to furnish **10** as a colorless oil (50 mg, yield of 36%). ^{31}P NMR (162 MHz, $CDCl_3$) δ = -4.0 (s, 1P). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.12-7.22 (m, 5H, Har), 6.82 (d, *J* = 8.5 Hz, 1H, Har), 6.76 (t, *J* = 5.9 Hz, 1H, NH), 5.94 (t, *J* = 1.3 Hz, 1H, Har), 5.57 (s, 1H), 4.50-4.56 (m, 2H), 3.88 (s, 3H), 3.85 (s, 3H), 3.64 (q, *J* = 6.6 Hz, 2H), 2.86 (t, *J* = 6.6 Hz, 2H), 2.46 (q, *J* = 7.4 Hz, 2H), 1.14 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.6 (C, C=O), 166.7 (C, C(=O)N), 154.5 (C, Car), 150.3 (C, Car), 149.6 (d, *J* = 6.9 Hz, C, Car), 135.3 (C, Car), 134.3 (C, Car), 131.6 (C, Car), 130.2 (2CH, CHar), 128.9 (CH_2, C=CH_2), 127.3 (C, Car), 123.0 (C, Car), 120.3 (d, *J* = 4.8 Hz, 2CH, Car), 110.9 (CH, Car), 68.2 (CH_2, OCH_2), 55.1 (d, *J* = 6.2 Hz, $2CH_3$), 40.2 (CH_2), 35.0 (CH_2), 23.5 (CH_2), 12.5 (CH_3). IR (neat): ν = 3416 (NH), 1666 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{23}H_{26}Cl_2NO_7P$: 530.0902, found: 530.0909.

4-(4-(2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetyl)piperazin-1-yl)phenyl dimethyl phosphate (11). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc/Et_3N, 80:20:0.5 (v/v/v) as eluent, to furnish **11** as a colorless oil (51 mg, yield of 39%). ^{31}P NMR (162 MHz, $CDCl_3$) δ = -3.6 (s, 1P). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.20-7.28 (m, 3H, Har), 6.99 (d, *J* = 8.6 Hz, 1H, Har), 6.87 (td, *J* =

9.3, 2.3 Hz, 2H, Har), 5.93 (t, *J* = 1.5 Hz, 1H), 5.58 (s, 1H), 4.86 (s, 2H), 3.86 (s, 3H), 3.83 (s, 3H), 3.75-3.81 (m, 4H), 3.14 (t, *J* = 5.0 Hz, 2H), 3.09 (t, *J* = 5.0 Hz, 2H), 2.46 (q, *J* = 7.4 Hz, 2H), 1.13 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.9 (C, C=O), 165.3 (C, C(=O)N), 155.3 (C, Car), 150.3 (C, Car), 148.4 (C, Car), 144.7 (d, *J* = 7.1 Hz, C, Car), 133.9 (C, Car), 131.6 (C, Car), 128.8 (CH_2, C=CH_2), 127.2 (CH, Car), 122.9 (C, Car), 120.7 (d, *J* = 4.7 Hz, 2CH, Car), 118.2 (2CH, Car), 110.8 (CH, Car), 68.9 (CH_2, OCH_2), 55.0 (d, *J* = 6.2 Hz, 2CH_3), 50.6 (CH_2), 50.0 (CH_2), 45.6 (CH_2), 42.3 (CH_2), 23.6 (CH_2), 12.5 (CH_3). IR (neat): ν = 3416 (NH), 1666 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{23}H_{26}Cl_2NO_7P$: 530.0902, found: 530.0909.

4-(2-(2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetamido)ethyl)phenyl diethyl phosphate (12). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 7:3 (v/v) as eluent, to furnish **12** as a colorless oil (103 mg, yield of 39%).^{31}P NMR (121 MHz, CD_2Cl_2) δ = -6.32 (s, P). ^{1}H NMR (300 MHz, CD_2Cl_2) δ = 7.15-7.29 (m, 5H, Har), 6.91 (d, *J* = 8.5 Hz, 1H, Har), 6.77 (s, 1H, NH), 6.00 (s, 1H), 5.60 (s, 1H), 4.57 (s, 2H), 4.14-4.29 (m, 4H), 3.65 (q, *J* = 6.9 Hz, 2H), 2.89 (t, *J* = 6.9 Hz, 2H), 1.17 (t, *J* = 7.4 Hz, 3H), 2.48 (q, *J* = 7.4 Hz, 2H), 1.37 (td, *J* = 7.1, 1.0 Hz, 6H). ^{13}C NMR (75 MHz, CD_2Cl_2) δ = 195.5 (C, C=O), 166.4 (C, C(=O)N), 154.6 (C, Car), 150.2 (C, Car), 149.6 (d, *J* = 6.8 Hz, Car), 135.3 (d, *J* = 1.2 Hz, Car), 133.9 (C, Car), 131.0 (2C, Car), 129.9 (C, Car), 128.6 (C, CH_2), 127.3 (C, Car), 125.4 (C, Car), 120.1 (d, *J* = 4.8 Hz, 2C, Car), 111.0 (C, Car), 68.2 (CH_2), 64.5 (d, *J* = 6.1 Hz, 2CH_3), 40.0 (CH_2), 34.8 (CH_2), 23.4 (CH_2), 15.9 (d, *J* = 6.6 Hz, 2CH_2), 12.2 (CH_3). IR (neat): ν = 3419 (NH), 1666 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{25}H_{30}Cl_2NO_7P$: 558.1215, found: 558.1227.

4-(4-(2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetyl)piperazin-1-yl)phenyl diethyl phosphate (13). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:1 to 1:1 (v/v) as eluent, to furnish **13** as a white solid (47 mg, yield of 46%).^{31}P NMR (162 MHz, $CDCl_3$) δ = -5.8 (s, 1P). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.09-7.18 (m, 3H, Har), 6.98 (d, *J* = 8.6 Hz, 1H, Har), 6.82-6.88 (m, 2H, Har), 5.93 (t, *J* = 1.5 Hz, 1H), 5.58 (s, 1H), 4.86 (s, 2H), 4.10-4.28 (m, 4H), 3.74-3.74 (m, 4H), 3.14 (t, *J* = 5.1 Hz, 2H), 3.09 (t, *J* = 5.1 Hz, 2H), 2.45 (q, *J* = 7.4 Hz, 2H), 1.34 (td, *J* = 7.1, 1.0 Hz, 6H), 1.13 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.9 (C, C=O), 165.3 (C, C(=O)N), 155.3 (C, Car), 150.3 (C, Car), 148.2 (C, Car), 144.9 (d, *J* = 7.0 Hz, C, Car), 133.9 (C, Car), 131.6 (C, Car), 128.8 (CH_2, C=CH_2), 127.2 (CH, Car), 122.9 (C, Car), 120.8 (d, *J* = 4.7 Hz, 2CH, Car), 118.2 (2CH, Car), 110.8 (CH, Car), 68.9 (CH_2, OCH_2), 64.6 (d, *J* = 6.1 Hz, 2CH_2), 50.7 (CH_2), 50.0 (CH_2), 45.6 (CH_2), 42.3 (CH_2), 23.5 (CH_2), 16.2 (d, *J* = 6.7 Hz, 2CH_3), 12.5 (CH_3). IR (neat): ν = 1663 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{27}H_{23}Cl_2N_2O_7P$: 599.1481, found: 599.1486.

2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-*N*-(4-((2-oxido-1,3,2-dioxaphosphinan-2-yl)oxy)phenethyl)acetamide (14). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 8:2 to 1:1 (v/v) as eluent, to furnish **14** as a colorless oil (75 mg, yield of 58%). ^{31}P NMR (162 MHz, CD_2Cl_2) δ = -13.4 (s, 1P). ^{1}H NMR (400 MHz, CD_2Cl_2) δ = 7.13-7.23 (m, 5H, Har), 6.81 (d, *J* = 6.1 Hz, 1H, Har), 6.77 (t, *J* = 8.5 Hz, 1H, NH), 5.94 (t, *J* = 1.3 Hz, 1H, Har), 5.57 (s, 1H), 4.51-4.58 (m, 6H), 3.63 (q, *J* = 6.6 Hz, 2H), 2.85 (t, *J* = 6.6 Hz, 2H), 2.45 (q, *J* = 7.4 Hz, 2H), 2.31-2.42 (m, 1H), 1.77-1.81 (m, 1H), 1.13 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, CD_2Cl_2) δ = 195.6 (C, C=O), 166.8 (C, C(=O)N), 154.6 (C, Car), 150.2 (C, Car), 149.4 (d, *J* = 6.7 Hz, C, Car), 135.2 (C, Car), 134.3 (C, Car), 131.5 (C, Car), 130.3 (2CH, CHar), 129.1 (CH_2, C=CH_2), 127.3 (C, Car), 123.1 (C, Car), 119.9 (d, *J* = 5.2 Hz, 2CH, Car), 110.9 (CH, Car), 69.5 (d, *J* =

7.4 Hz, $2CH_2$, OCH_2), 68.3 (CH_2, OCH_2), 40.3 (CH_2), 34.9 (CH_2), 26.2 (d, *J* = 7.3 Hz, CH_2), 23.5 (CH_2), 12.52 (CH_3). IR (neat): ν = 3418 (NH), 1664 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{24}H_{26}Cl_2NO_7P$: 542.0902, found: 542.0911.

1-(2,3-dichloro-4-(2-(4-(4-((2-oxido-1,3,2-dioxaphosphinan-2-yl)oxy)phenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)-2-methylenebutan-1-one (15). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:1 to 1:1 (v/v) as eluent, to furnish **14** as a white solid (60 mg, yield of 59%). ^{31}P NMR (162 MHz, $CDCl_3$) δ = -13.0 (s, 1P). ^1H NMR (400 MHz, $CDCl_3$) δ = 7.12-17.19 (m, 3H, Har), 6.99 (d, *J* = 8.6 Hz, 1H, Har), 6.84-6.91 (m, 2H, Har), 5.93 (s, 1H), 5.59 (s, 1H), 4.86 (s, 2H), 4.42-4.55 (m, 4H), 3.74-3.82 (m, 4H), 3.14 (t, *J* = 5.1 Hz, 2H), 3.09 (t, *J* = 5.1 Hz, 2H), 2.46 (q, *J* = 7.4 Hz, 2H), 2.30-2.42 (m, 1H), 1.75-1.86 (m, 1H), 1.14 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.9 (C, C=O), 165.4 (C, C(=O)N), 155.4 (C, Car), 150.3 (C, Car), 148.5 (C, Car), 144.5 (d, *J* = 6.8 Hz, C, Car), 133.9 (C, Car), 131.7 (C, Car), 128.7 (CH_2, C=CH_2), 127.3 (CH, Car), 122.9 (C, Car), 120.6 (d, *J* = 5.0 Hz, 2CH, Car), 118.5 (2CH, Car), 110.9 (CH, Car), 69.4 (d, *J* = 7.3 Hz, $2CH_2$), 68.9 (CH_2, OCH_2), 50.7 (CH_2), 50.1 (CH_2), 45.7 (CH_2), 42.3 (CH_2), 26.1 (d, *J* = 7.3 Hz, CH_2), 23.6 (CH_2), 12.6 (CH_3). IR (neat): ν = 1661 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{26}H_{29}Cl_2N_2O_7P$: 583.1168, found: 583.1177.

N-(4-((4-(3-chlorophenyl)-2-oxido-1,3,2-dioxaphosphinan-2-yl)oxy)phenethyl)-2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetamide (16). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:1 to 1:1 (v/v) as eluent, to furnish **16** as a white foam (40 mg, yield of 50%). ^{31}P NMR (162 MHz, $CDCl_3$) δ= -11.3 (s, 1P). ^1H NMR (400 MHz, $CDCl_3$) δ = 7.29-7.31 (m, 2H, Har), 7.25-7.28 (m, 1H, Har), 7.15-7.24 (m, 6H, Har), 6.76-6.83 (m, 2H, Har), 5.94 (t, *J* = 1.5 Hz, 1H, Har), 5.60-5.74 (m, 1H), 5.56 (s, 1H), 4.62-4.78 (m, 1H), 4.45-4.56 (m, 3H), 3.65 (q, *J* = 6.7 Hz, 2H), 2.88 (t, *J* = 6.7 Hz, 2H), 2.46 (q, *J* = 7.4 Hz, 2H), 2.24-2.38 (m, 1H), 2.06-2.18 (m, 1H), 1.14 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.7 (C, C=O), 166.8 (C, C(=O)N), 154.5 (C, Car), 150.4 (C, Car), 149.6 (d, *J* = 7.5 Hz, C, Car), 140.7 (d, *J* = 7.9 Hz, C, Car), 135.7 (d, *J* = 1.3 Hz, C, Car), 134.9 (C, Car), 134.4 (C, Car), 131.6 (C, Car), 130.2 (CH, CHar), 130.2 (2CH, CHar), 129.1 (CH, CHar), 128.9 (CH_2, C=CH_2), 127.3 (CH, Car), 125.9 (CH, Car), 123.8 (CH, Car), 123.1 (C, Car), 120.7 (d, *J* = 4.7 Hz, 2CH, Car), 110.9 (CH, Car), 80.1 (d, *J* = 5.5 Hz, CH), 68.3 (CH_2, OCH_2), 67.3 (d, *J* = 5.8 Hz, CH_2), 40.3 (CH_2), 35.0 (CH_2), 33.4 (d, *J* = 7.4 Hz, CH_2), 23.6 (CH_2), 12.5 (CH_3). IR (neat): ν = 3419 (NH), 1666 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{30}H_{29}Cl_3NO_7P$: 652.0825, found: 652.0851.

1-(2,3-dichloro-4-(2-(4-(4-((4-(3-chlorophenyl)-2-oxido-1,3,2-dioxaphosphinan-2-yl)oxy)phenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)-2-methylenebutan-1-one (17). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 1:1 (v/v) as eluent, to furnish **17** as a white foam (60 mg, yield of 67%). ^{31}P NMR (162 MHz, $CDCl_3$) δ = -10.9 (s, 1P). ^1H NMR (400 MHz, $CDCl_3$) δ = 7.27-7.33 (m, 2H, Har), 7.24-7.27 (m, 1H, Har), 7.11-7.18 (m, 4H, Har), 6.99 (d, *J* = 8.6 Hz, 1H, Har), 6.86-6.91 (m, 2H, Har), 5.93 (t, *J* = 1.5 Hz, 1H, Har), 5.61-5.69 (m, 1H), 5.58 (s, 1H), 4.86 (s, 2H), 4.61-4.77 (m, 1H), 4.40-4.54 (m, 1H), 3.78 (q, *J* = 5.3 Hz, 4H), 3.06-3.22 (m, 4H), 2.45 (q, *J* = 7.4 Hz, 2H), 2.15-2.37 (m, 1H), 2.02-2.20 (m, 1H), 1.13 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 195.9 (C, C=O), 165.4 (C, C(=O)N), 155.4 (C, Car), 150.3 (C, Car), 148.7 (C, Car), 144.6 (d, *J* = 7.8 Hz, C, Car), 140.9 (d, *J* = 8.0 Hz, C, Car), 134.9 (C, Car), 133.9 (C, Car), 131.6 (C, Car), 130.3 (2CH, CHar), 129.1 (CH, CHar), 128.8 (CH_2, C=CH_2), 127.3 (CH, Car), 125.9 (CH, Car), 123.8 (CH, Car), 122.9 (C, Car), 121.1 (d, *J* = 4.5 Hz, 2CH, Car), 118.2 (CH, Car), 110.8 (CH, Car), 80.1 (d, *J* = 5.5 Hz, CH), 68.9 (CH_2, OCH_2), 67.4 (d, *J* =

5.8 Hz, CH_2), 50.5 (CH_2), 49.9 (CH_2), 45.5 (CH_2), 42.3 (CH_2), 33.5 (d, *J* = 7.2 Hz, CH_2), 23.5 (CH_2), 12.6 (3). IR (neat): ν = 1663 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{32}H_{32}Cl_3N_2O_7P$: 693.1091, found 693.1124. Elemental analysis calculated (%) for $C_{32}H_{32}Cl_3N_2O_7P$: C 55.39, H 4.65, N 4.04, found: C 55.49, H 4.38, N 3.77.

4-(2-(2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetamido)ethyl)-2-methoxyphenyl diethyl phosphate (20). Following general procedure, the crude residue was purified by flash chromatography, using DCM/EtOAc, 2:8 (v/v) as eluent, to furnish **20** as a white foam (122 mg, yield of 47%). ^{31}P NMR (121 MHz, CD_2Cl_2) δ = -5.85 (s, P). ^1H NMR (300 MHz, CD_2Cl_2) δ = 7.22 (d, *J* = 8.6 Hz, 1H, Har), 7.21 (dd, *J* = 8.1, 1.9 Hz, 1H, Har), 6.91 (d, *J* = 8.6 Hz, 1H, Har), 6.89 (s, 1H), 6.84 (s, 1H, NH), 6.80 (dd, *J* = 8.1, 1.9 Hz, 1H), 5.99 (s, 1H), 5.60 (s, 1H), 4.57 (s, 2H), 4.16-4.31 (m, 4H), 3.88 (s, 3H, OCH_3), 3.66 (q, *J* = 6.9 Hz, 2H), 2.89 (t, *J* = 7.0 Hz, 2H), 2.48 (q, *J* = 7.4 Hz, 2H), 1.37 (td, *J* = 7.1, 1.0 Hz, 6H), 1.17 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, CD_2Cl_2) δ = 195.3 (C, C=O), 166.4 (C, C(=O)N), 154.6 (C, Car), 150.7 (d, *J* = 5.3 Hz, C, Car), 150.2 (C, Car), 138.6 (d, *J* = 7.1 Hz, C, Car), 136.4 (d, *J* = 1.5 Hz, C, Car), 134.0 (C, Car), 131.0 (C, Car), 128.6 (CH_2, C=CH_2), 127.3 (CH, Car), 122.7 (C, Car), 121.2 (d, *J* = 2.8 Hz, CH, Car),120.6 (d, *J* = 1.3 Hz, CH, Car), 113.1 (CH, Car), 111.1 (CH, Car), 68.3 (CH_2), 64.5 (d, *J* = 6.1 Hz, $2CH_2$), 55.9 (OCH_3), 39.9 (CH_2), 35.3 (CH_2), 23.4 (CH_2), 15.9 (d, *J* = 6.8 Hz, $2CH_3$),12.2 (C, CH_3). IR (neat): ν = 3419 (NH), 1666 (C=O) cm^{-1}.

4-hydroxyphenethyl 2-(2,3-dichloro-4-(2-methylenebutanoyl) phenoxy)acetate (21).To a mixture of paratoluenesulfonic acid (APTS) (1.1 equiv) and ethacrynic acid (1 equiv) in DCM (10 mL) was added at 0°C 4-(2-hydroxyethyl)phenol (1 equiv). The reaction mixture was heated to 50 °C for 12h and then evaporated to dryness. The crude residue was purified by flash chromatography using hexane/EtOAc, 4:6 (v/v) as eluent, to furnish **21** as a white solid (256 mg, yield of 92%). ^1H NMR (400 MHz, CD_2Cl_2) δ = 7.08 (d, *J* = 8.6 Hz, 1H, Har), 7.01 (d, *J* = 8.4 Hz, 2H, Har), 6.69 (d, *J* = 8.4 Hz, 2H, Har), 6.62 (d, *J* = 8.6 Hz, 1H, Har), 6.02 (s, 1H), 5.63 (s, 1H), 5.54 (s, 1H, OH), 4.76 (s, 2H), 4.40 (t, *J* = 6.5 Hz, 2H), 2.91 (t, *J* = 6.5 Hz, 2H), 2.49 (q, *J* = 7.4 Hz, 2H), 1.18 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, CD_2Cl_2) δ = 196.3 (C, C=O), 167.7 (C, C(=O)N), 155.5 (C, Car), 154.6 (C, Car), 150.3 (C, Car), 133.5 (C, Car), 131.1 (C, Car), 129.8 (2CH, Car), 129.3 (C, Car), 129.2 (CH_2, C=CH_2), 126.8 (CH, Car), 122.7 (C, Car), 115.4 (2CH, Car), 110.4 (CH, Car), 66.2 (CH_2), 66.1 (CH_2), 33.9 (CH_2), 23.4 (CH_2), 12.2 (CH_3). IR(neat): ν = 3438 (NH), 1750 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{21}H_{20}Cl_2O_5$: 423.0766, found: 423.0773.

2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-*N*-(4-hydroxy-3-methoxyphenethyl) acetamide (18).To a mixture of DCC (1.1 equiv), HOBt (1.1 equiv) and ethacrynic acid (1 equiv) in dry DMF (5 mL) were added at 0°C, 4-(2-aminoethyl)-2-methoxyphenol (1 equiv). The reaction mixture was stirred overnight and then the solution was filtered and evaporated. The product was purified by flash chromatography using DCM/EtOAc, 3:7 (v/v) as eluent, to furnish **18** as a white solid (143 mg, 48%). ^1H NMR (300 MHz, CD_2Cl_2) δ = 7.20 (d, *J* = 8.5 Hz, 1H, Har), 7.00 (s, 1H, Har), 6.66-6.91 (m, 4H, Har and NH), 5.99 (s, 1H, C=CH_2), 5.60 (s, 1H, C=CH_2), 5.20 (s, 1H, OH), 4.56 (s, 2H, OCH_2), 3.85 (s, 3H, OCH_3), 3.62 (dd, *J* = 12.3, 6.3 Hz, 2H, CH_2), 2.82 (t, *J* = 6.9 Hz, 2H, CH_2), 2.48 (q, *J* = 7.4 Hz, 2H, CH_2), 1.17 (t, *J* = 7.4 Hz, 1H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 195.39 (C, C=O), 166.39 (C, C(=O)N), 154.59 (CH, Car), 150.16 (C, Car), 147.10 (C, Car), 144.98 (C, Car), 135.82 (C, Car), 133.92 (C, Car), 131.02 (C, Car), 129.87 (C, Car), 128.66 (CH_2, C=CH_2), 127.24 (CH, Car), 121.29 (CH, Car) , 114.71 (CH, Car), 111.41 (CH, Car), 110.98 (CH, Car), 68.28 (CH_2), 55.79 (OCH_3), 40.20 (CH_2), 35.05 (CH_2), 23.38 (CH_2), 12.20 (CH_3).

General Procedure: Preparation of **23**, **25** and **26**. To a solution of ethacrynic acid analog **3** (1 equiv) and amine (1-(4-methoxyphenyl)piperazine, (1-(4-hedroxyphenyl)piperazine or tyramine) (1 equiv) in a mixture of DCM/DMF (5 ml), EDCI (1.5 equiv) and a catalytic amount of DMAP were added. The reaction mixture was stirred overnight at room temperature, then, ethyl acetate (50 ml) was added and the organic layer was washed with water (2 x 50 ml) and brine (3 x 50 ml). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by column chromatography.

2-(2,3-dichloro-4-(2-methylbutanoyl)phenoxy)-*N*-(4-hydroxyphenethyl)acetamide (23). Following general procedure, the crude residue was purified by column chromatography, using DCM/EtOAc, 8:2 (v/v) as eluent, to furnish **23** as a white foam (50 mg, yield of 51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.22 (d, *J* = 8.6 Hz, 1H, Har), 7.00 (td, *J* = 8.6, 1.4 Hz, 2H, Har), 6.68-6.75 (m, 3H), 6.61 (t, *J* = 5.8 Hz, 1H, NH), 5.78 (br s, 1H, OH), 4.54 (s, 2H), 3.62 (q, *J* = 6.6 Hz, 2H), 3.12-3.23 (m, 1H), 2.79 (t, *J* = 6.6 Hz, 2H), 1.72-1.88 (m, 1H), 1.38-1.51 (m, 1H), 1.17 (d, *J* = 6.9 Hz, 3H), 0.93 (t, *J* = 7.4, Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 206.8 (C, C=O), 166.9 (C, C(=O)N), 154.9 (C, Car), 154.8 (C, Car), 135.3 (C, Car), 131.3 (C, Car), 129.84 (C, Car), 129.79 (2CH, Car), 127.2 (CH, Car), 123.3 (C, Car), 115.9 (2CH, Car), 110.8 (CH, Car), 68.1 (CH_2, OCH_2), 47.1 (CH), 40.3 (CH_2), 34.4 (CH_2), 26.0 (CH_2), 15.7 (CH_3), 11.7 (CH_3). IR (neat): ν = 3402 (OH), 1664 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{21}H_{23}Cl_2NO_4$: 424.1082, found: 424.1075.

1-(2,3-dichloro-4-(2-(4-(4-hydroxyphenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)-2-methylbutan-1-one (25). Following general procedure, the crude residue was purified by column chromatography, using DCM/EtOAc, 8:2 to 5:5 (v/v) as eluent, to furnish **25** as a white foam (58 mg, yield of 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.26 (d, *J* = 8.6 Hz, 1H, Har), 6.98 (d, *J* = 8.6 Hz, 1H, Har), 6.74-6.85 (m, 4H, Har), 5.31 (br s, 1H, OH), 4.86 (s, 2H), 3.77 (t, *J* = 5.0 Hz, 4H), 3.17 (h, *J* = 6.8 Hz, 1H), 3.03 (td, *J* = 21.0, 5.0 Hz, 4H), 1.77 (dqd, *J* = 13.5, 7.5, 6.0 Hz, 1H), 1.43 (pd, *J* = 14.5, 7.5 Hz, 1H), 1.16 (d, *J* = 6.9 Hz, 3H), 0.91 (t, *J* = 7.4, Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 206.3 (C, C=O), 165.4 (C, C(=O)N), 155.7 (C, Car), 150.8 (C, Car), 145.1 (C, Car), 135.0 (C, Car), 131.4 (C, Car), 127.3 (CH, Car), 123.3 (C, Car), 119.4 (2CH, Car), 116.1 (2CH, Car), 111.0 (CH, Car), 68.8 (CH_2, OCH_2), 51.7 (CH_2), 51.0 (CH_2), 46.9 (CH), 45.8 (CH_2), 42.6 (CH_2), 26.1 (CH_2), 15.8 (CH_3), 11.7 (CH_3). IR (neat): ν = 3320 (OH), 1692 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{23}H_{26}Cl_2N_2O_4$ 465.1348, found 465.1339. Elemental analysis calculated (%) for $C_{23}H_{26}Cl_2N_2O_4$: C 59.36, H 5.63, N 6.02, found: C 59.36, H 5.46, N 5.84.

1-(2,3-dichloro-4-(2-(4-(4-methoxyphenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)-2-methylbutan-1-one (26). Following general procedure, the crude residue was purified by column chromatography, using DCM/EtOAc, 8:2 (v/v) as eluent, to furnish **26** as a white foam (64 mg, yield of 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.27 (d, *J* = 8.7 Hz, 1H, Har), 6.99 (d, *J* = 8.7 Hz, 1H, Har), 6.82-6.92 (m, 4H, Har), 4.87 (s, 2H), 3.75-3.80 (m, 7H), 3.14-3.25 (m, 1H), 3.01-3.13 (m, 4H), 1.72-1.86 (m, 1H), 1.37-1.52 (m, 1H), 1.16 (d, *J* = 6.9 Hz, 3H), 0.92 (t, *J* = 7.4, Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 206.2 (C, C=O), 165.2 (C, C(=O)N), 155.8 (C, Car), 154.7 (C, Car), 145.2 (C, Car), 135.0 (C, Car), 131.4 (C, Car), 127.3 (2CH, Car), 123.3 (C, Car), 119.2 (CH, Car), 114.7 (2CH, Car), 111.0 (CH, Car), 68.9 (CH_2, OCH_2), 55.7 (CH_3, OCH_3), 51.6 (CH_2), 51.0 (CH_2), 46.9 (CH), 45.8 (CH_2), 42.5 (CH_2), 26.1 (CH_2), 15.8 (CH_3), 11.8 (CH_3). IR (neat): ν = 1692 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{24}H_{28}Cl_2N_2O_4$: 479.1504, found: 479.1499.

2-(2,3-dichloro-4-(1-hydroxy-2-methylenebutyl)phenoxy)-*N*-(4-hydroxyphenethyl)acetamide (24). To a mixture of compound 4 (1 equiv) and tyramine (1 equiv) in a mixture of DCM/DMF (30 ml), EDCI (1.5 equiv) and a catalytic amount of DMAP were added. The reaction mixture was stirred overnight at room temperature, then, ethyl acetate (50 ml) was added and the organic layer was washed with water (2 x 50 ml) and brine (3 x 50 ml). The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The obtained residue was purified by column chromatography using DCM/EtOAc, 8:2 to 5.5 (v/v) as eluent, to furnish **24** as a white foam (70 mg, yield of 15%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.32 (d, *J* = 8.7 Hz, 1H, Har), 6.97 (d, *J* = 8.5 Hz, 2H, Har), 6.64-6.73 (m, 4H), 6.51 (br s, 1H, OH), 5.55 (s, 1H), 5.15 (s, 1H), 5.03 (s, 1H), 4.49 (d, *J* = 14.8 Hz, 1H), 4.43 (d, *J* = 14.8 Hz, 1H), 3.50-3.70 (m, 2H), 2.68-2.87 (m, 3H), 1.85-2.04 (m, 2H), 1.02 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 167.7 (C, C(=O)N), 154.9 (C, Car), 153.0 (C, Car), 151.0 (C, Car), 134.9 (C, Car), 133.1 (C, Car), 129.8 (2CH, CHar), 129.7 (C, Car), 126.8 (CH, Car), 122.1 (C, Car), 115.9 (2CH, Car), 111.3 (CH, Car), 110.4 (CH_2, C=CH_2), 73.4 (CH), 68.0 (CH_2, OCH_2), 40.4 (CH_2), 34.3 (CH_2), 25.3 (CH_2), 12.2 (CH_3). IR (neat): ν =3400 (OH), 1662 (C=O) cm^{-1}. HRMS $[M+H]^+$ calculated for $C_{21}H_{23}Cl_2NO_4$: 424.1082, found: 424.1066.

Cell culture. The human cell lines KB (nasopharyngeal epidermis carcinoma) and SK-OV-3 (ovary adenocarcinoma) were originated from the NCI, HCT116 (colon adenocarcinoma) and PC3 (prostate adenocarcinoma) were purchased from ECACC (Salisbury, UK), EPC (carp skin epithelium), MRC5-SV2 (human fetal lung) and HL60 (promyeocytic leukaemia) cells from ATCC. MCF7 (breast adenocarcinoma) were kindly provided by Dr M. Kassack (Bonn University, Germany) and OVCAR8 (ovary carcinoma) by Dr M. Liscovitch (Rehovot, Israel). KB and MRC5 cells were grown in D-MEM medium supplemented with 10% fetal calf serum, in the presence of penicillin, streptomycin and fungizone in 75 cm^2 flask under 5% CO_2, whereas all other cell lines were grown in complete RPMI medium.

Cell proliferation assay. Cells were plated in 96-well tissue culture plates in 200 µL medium and treated 24 h later with compounds dissolved in DMSO. Control cells received the same volume of DMSO (1% final volume). After 24 or 72h exposure to the drug, inhibition of cell proliferation was determined as previously reported [21]. For IC_{50} determinations (50% inhibition of cell proliferation) experiments were performed in duplicate with concentrations ranged 0.5 nm to 100 µm.

Cell viability. Cell viability and GSH content were measured in HL60 cells treated with compounds for 24h. 100,000 cells were grown in 500 µl medium in 24-well microplates: viability was monitored on a 20 µl aliquot by flow cytometry after addition of the Viacount reagent following the recommendations of the manufacturer and fluorescence was measured with a Guava EasyCyte plus cytometer (Guava, Merck-Millipore, Billerica, USA). Remaining cells were pelleted by gentle centrifugation, resuspended in 250 µl phosphate buffered solution and sonicated. Lysate (20 µl) was transferred to a 384-well black microplate and incubated in a 50 µl final volume of 100 mM sodium phosphate (pH 7.5) with 100 µM monochlorobimane and 0.4 µg recombinant human GST π (Oxford Biomedical Research, Oxford, USA). Fluorescence was monitored (excitation 380 nm, emission 460 nm) over a 60 min period.

Necrosis. Necrosis was estimated through the release of LDH in the culture medium. 20,000 HL60 cells were incubated for 24 and 48 h in the presence of chemicals in 96-well [21,31]. Fluorescence was recorded (exc 560 nm, em 590 nm): results are expressed as the residual

activity in the presence of chemicals compared to activity in the presence of vehicle alone. 50 µM Menadione was used as positive control.

Flow cytometric detection of apoptosis. Apoptotic and necrotic cells were analysed in flow cytometry using HL60 cells [21,31]. The cellular fluorescence was measured by flow cytometry with a Guava EasyCyte plus cytometer (Millipore).

Caspase activity assay. Caspase activities were assayed in HL60 cell after a 24h treatment with compounds [21,31]. Fluorescence was recorded (exc 360 nm, em 435 nm) after 0, 30, 60, 120, and 180 min. Reaction rates were calculated from the slope of the linear time-dependent reaction and are expressed as the fold-activation relative to the control (HL60 with DMSO alone). 50 nm Doxorubicin was used as a positive control.

Cell cycle analysis. HL60 cells (25,000 cells/well in 96-well microplates) were exposed for 24 and 48 h as reported elsewhere [21,31]. Controls received the same volume of DMSO (1% final volume). Cells were analyzed by flow cytometry with a Guava Easycyte cytometer (Millipore) and cell populations were quantified using Modfit LT (Verity Software House).

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6. References

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Highlights

- New ethacrynic acid derivatives were found as efficient antiproliferative agents

- Anti-proliferative activities increased by substitution of the carboxylic function of the classical ethacrynic acid by hydrophobic aryl-alkyl(cycloalkyl) chains

- More complex mechanism of action than the simple inhibition of glutathione S-transferase observed with ethacrynic acid was pointing out.