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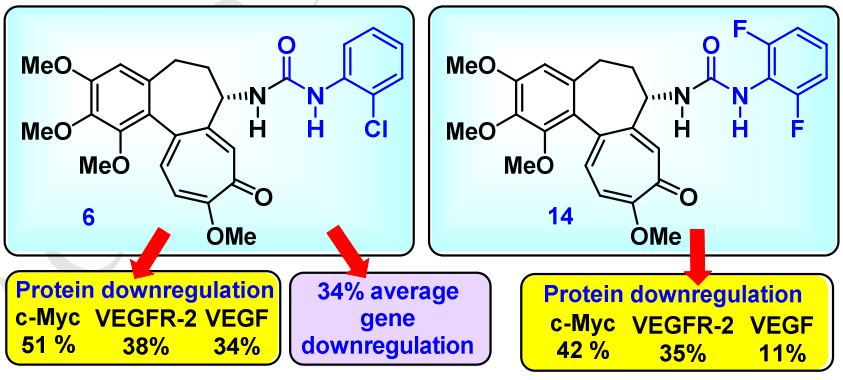
Graphical Abstract

Twenty-seven *N,N´*-disubstituted ureas containing a colchicine moiety an and aryl fragment have been synthetized and biologically evaluated. The cytotoxicity of the compounds, their ability to inhibit the expression of oncogenes related to telomerase activation and to the VEGF/VEGFR-2 autocrine process, such as *c-MYC*, *hTERT* and *VEGF* and their capability to downregulate c-MYC and VEGFR-2 proteins and the secretion of VEGF have been measured. In these biological evaluations, we have found that the change of the acetyl group in colchicines for an *N*-arylurea unit causes a great improvement in anticancer properties. The most promising derivatives were compounds **6** (*o*-Cl) and **14** (*o,o*-di-F) as they were able to downregulate all the tested targets at a concentration below their IC_{50} values. Thus, the arylurea unit enhances the potential of colchicine as an anticancer agent.



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Keywords: colchicine, urea derivatives, cytotoxicity, oncogenes downregulation

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Arylureas derived from colchicine: Enhancement of colchicine oncogene downregulation activity

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ABSTRACT

Our efforts to get therapeutically useful colchicine derivatives for the treatment of cancer have led us to synthetize and biologically evaluate twenty-seven *N*,*N´*-disubstituted ureas containing a colchicine moiety an and aryl fragment. The cytotoxicity of the compounds, their ability to inhibit the expression of oncogenes related to telomerase activation and to the VEGF/VEGFR-2 autocrine process, such as *c-MYC*, *hTERT* and *VEGF* and their capability to downregulate c-MYC and VEGFR-2 proteins and the secretion of VEGF have been measured. In these biological evaluations, we have found that the change of the acetyl group in colchicines for an *N*-arylurea unit causes a great improvement in anticancer properties. The most promising derivatives were compounds **6** (*o*-Cl) and **14** (*o*,*o*-di-F) as they were able to downregulate all the tested targets at a concentration below their IC_{50} values. Thus, the arylurea unit enhances the potential of colchicine as an anticancer agent.

1. Introduction

During eons Nature has been producing small-molecule products able to interact within living organisms with macromolecular targets that contain structural domains similar to human proteins. As a result of this natural selection process, a vast range of structures has arisen that have been found to show optimal interactions with many biological targets [1]. Not surprisingly, natural product research is a growing domain at the interface of chemistry and biology which has not ceased to generate new chemical entities with high pharmacological interest [2]. In the last years, we have been investigating a range of analogues of natural products [3] for their potential value in anticancer therapy [4]. It is widely known that a range of both external and internal factors may induce cancer. This has led to the development of various types of therapeutic approaches [5]. One of them involves the use of cytotoxic drugs, which lead in many cases to cell apoptosis [6]. A second type is based on the use of compounds with vascular-targeting properties, either through inhibition of the formation of new blood vessels (antiangiogenic agents) or by means of destruction of existing ones (antivascular agents) [7]. Tumor angiogenesis is a complex process and involves

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the tight interplay of many factors [8]. One of them is the vascular endothelial growth factor (VEGF), a key regulator of angiogenesis that promotes endothelial cell survival, proliferation and migration while increasing vascular permeability through the interaction with VEGFR-2, a transmembrane protein kinase [9]. It is worth noting that overexpression in the production of VEGF has been found to occur in various types of tumors [10]. In addition, though VEGFRs were initially found on endothelial cells, recent studies have demonstrated that tumor cells of different origins also express VEGFRs as well as the existence of autocrine VEGF/VEGFR-2 signalling pathways in such cells. It is the presence of autocrine VEGF loops on tumor cells themselves that allows the latter to mediate their own survival, invasiveness and migration through VEGF pathways. This has converted VEGF and its receptor VEGFR-2 into key target molecules for cancer therapy [11,12].

A third therapeutic line follows the path of the chromosomal telomeres, the terminal zones of chromosomes that, due to their special structure, are recognized as functional domains and thus distinguished from random chromosomal breaks that would stimulate the onset of repair mechanisms. The aforementioned structure is maintained by a special type of ribonucleoprotein complex called telomerase, the expression of which is restricted or absent in normal human somatic cells. Telomeres progressively shorten during cell lifespan [13]. For this reason, and since telomerase has been detected in about 90% of all malignant tumors, drugs with ability to inhibit telomerase activity are potentially useful weapons in the fight against cancer, aging and other diseases related to premature telomere shortening [14].

Telomerase activity is positively associated with high levels of *hTERT* expression. A correlation between *hTERT* and *VEGF* expression has been established suggesting the existence of an autoregulatory loop between *VEGF* and *hTERT*. Further, expression of both *hTERT* and *VEGF* is linked to tumour progression and shows a positive association with *c*-MYC, a transcription factor that regulates cell proliferation, differentiation and promotes changes in the tumor microenvironment [15].

The discovery and investigation of new drugs therefore constitutes an important goal in chemistry and pharmacology. The emergence of resistances to existing drugs has led to a continuous need of developing new bioactive compounds able to overcome such problems. Even though first observed in the case of antibiotics, resistances have been reported to cancer therapies with various types of cytotoxic [16], antiangiogenic [17] and telomerase inhibitory agents [18].

Our interest in natural product analogues has led us to prepare several structural classes and to investigate their potential activity in the aforementioned types of biological activity. Among the natural products that we have included in our research, colchicine has particularly called our attention (see Figure 1). Surprisingly, even though it is one of the oldest drugs still currently available, little is known about its precise mode of action. Beyond its present medicinal use (gout,

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among other diseases), numerous other conditions have been recently proposed for the use of this drug. The marked toxicity of the compound, however, has prevented its use in cancer therapy [19]. Recently, we have published two reports on the biological properties of several colchicine analogues [4,20]. The properties investigated included not only the cytotoxicity of the compounds but also their ability to inhibit the expression of certain genes related to the angiogenesis process [21] and to telomerase activation [22].

It is worth noting that protein kinases, such as VEGFR-2, have emerged as one of the most successful families of drug targets [23]. It is thus not surprising that many thousands of inhibitors of several hundreds of kinases have been identified to date [24]. In fact, one of these inhibitors, sorafenib (see Figure 1), called our attention because of its activity against VEGFR-2, one of the main topics of interest of our research. Sorafenib is an oral multikinase inhibitor with anticancer activity against a wide spectrum of cancers and has been approved for the treatment of patients with various types of cancer [25]. Sorafenib contains a *N*,*N´*-bisaryl urea moiety with two different arylic fragments. Indeed, *N*-substituted urea derivatives of various structural types represent one highly important class of biologically active agents [26].

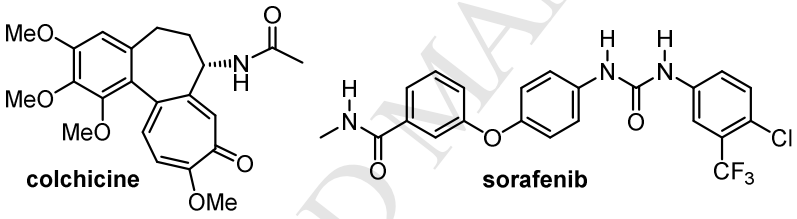

Figure 1. Structure of the colchicine and sorafenib

In recent years, many reports have been published on synthetic and mechanistic aspects of the aforementioned compound class, the evaluation of their various biological activities and the establishment of structure-activity relationships (SAR) [27]. Urea bioisosteres have also been investigated in SAR studies for their medicinal applications [28].

Focused on our research on novel natural product analogues with potential utility in cancer therapy [4], and on the basis of the aforementioned aspects, we conceived the synthesis and biological evaluation of a set of *N*,*N´*-disubstituted ureas containing one aryl fragment bound to one the two nitrogen atoms and one colchicine moiety connected to the other nitrogen. Thus, compounds **1**-**26** (see figure 2) were prepared and subsequently investigated for their biological activity. For the sake of estimation of the importance of the aryl moiety, compound **27**, which has an *N*-cyclohexyl group, was also prepared.

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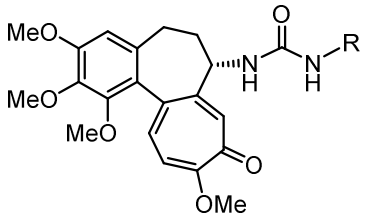
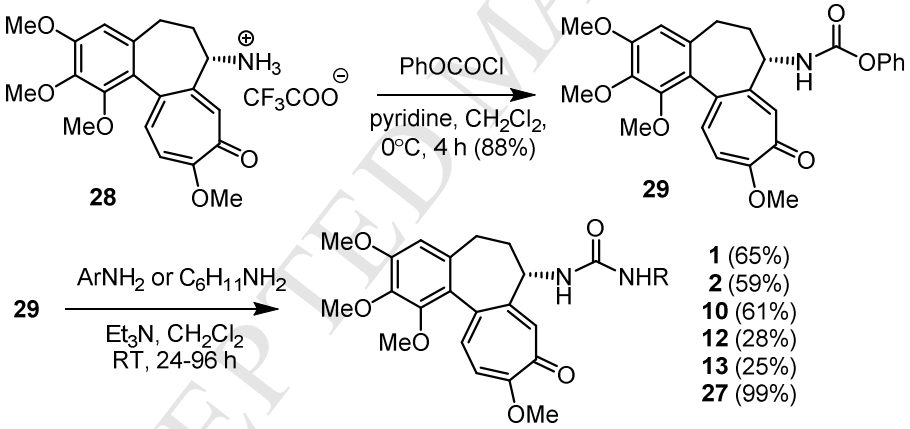


1 R = C_6H_5
2 R = *o*-MeC_6H_5
3 R = *o*-$CF_3C_6H_5$
4 R = *o*-$OMeC_6H_5$
5 R = *o*-FC_6H_5
6 R = *o*-ClC_6H_5
7 R = *o*-CNC_6H_5
8 R = *o*-$CF_3OC_6H_5$
9 R = *m*-ClC_6H_5
10 R = *p*-$MeOC_6H_5$
11 R = *p*-FC_6H_5
12 R = *o,o*-diMeC_6H_4
13 R = *o,o*-diEtC_6H_4
14 R = *o,o*-diFC_6H_4
15 R = *o,o*-diClC_6H_4
16 R = *o*-Me-*p*-FC_6H_4
17 R = *o*-Cl-*p*-FC_6H_4
18 R = *o*-Cl-*p*-MeC_6H_4
19 R = *o*-Cl-*p*-$CF_3C_6H_4$
20 R = *o,p*-diClC_6H_4
21 R = *m,m*-diFC_6H_4
22 R = *m,m*-di$CF_3C_6H_4$
23 R = *o,o*-diCl-*p*-FC_6H_3
24 R = *p*-$C_6H_5C_6H_5$
25 R = 1-naphthyl
26 R = 2-naphthyl
27 R = cyclohexyl

Figure 2. Structure of the colchicine analogues investigated in this study

2. Synthesis

The urea derivatives were prepared from the trifluoroacetate salt of *N*-deacetyl colchicine **28** [29] as depicted in Scheme 1. Thus, treatment of **28** with phenyl chloroformiate gave urethane **29** with good yield. Subsequently, **29** was allowed to react with a range of aromatic amines to yield the desired ureas via nucleophilic substitution of the phenoxy group [30]. However, acceptable yields were observed only in the case of aniline derivatives substituted with electron-donating groups. The only nonaromatic amine, cyclohexylamine, gave an excellent yield of the corresponding urea derivative **27** (see scheme 1).



Scheme 1. Synthesis of colchicine derivatives from aniline substituted with electron-donating groups and **27**

Anilines with electron-withdrawing substituents reacted very slowly and with poor yields. Under forcing conditions, products of nucleophilic attack at the tropolone ring were observed [31]. In order to avoid this problem, we decided to invert the order of formation of the two C−N bonds, with deacetyl colchicine being now the nucleophilic component of the reaction. For this purpose, the less reactive anilines were transformed into their carbamates by means of treatment with phenyl chloroformate. The latter were then allowed to react with **28** under basic conditions (pyridine, 80°C) to yield the desired urea derivatives with the yields indicated in scheme 2.

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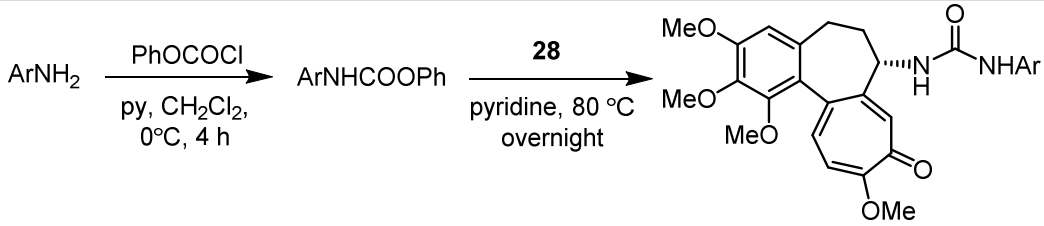

3 (16%) **11** (38%) **20** (38%)
4 (41%) **14** (46%) **21** (38%)
5 (53%) **15** (27%) **22** (22%)
6 (69%) **16** (47%) **23** (32%)
7 (60%) **17** (55%) **24** (31%)
8 (15%) **18** (88%) **25** (32%)
9 (32%) **19** (47%) **26** (27%)

Scheme 2. Synthesis of colchicine derivatives from aniline substituted with electron-withdrawing groups

3. Biological results

3.1. Effect on the inhibition of cell proliferation

The capability of our derivatives **1**-**27** to inhibit cell proliferation was measured by means of their IC_{50} values towards the cancer cell lines HT-29, MCF-7 and HeLa and towards one non-tumor cell line HEK-293. The achieved results are shown in Table 1.

Table 1. IC_{50} values (nM) of synthetic compounds **1**-**27** in cancer cell lines HT-29, MCF-7 and A549, and one non-cancer cell line HEK-293.[a]

Compound	HT-29	MCF-7	HeLa	HEK-293
Colchicine	13 ± 2	20 ± 2	14 ± 1	13 ± 3
1	11 ± 2	12 ± 3	12 ± 2	10 ± 5
2	26 ± 2	119 ± 8	32 ± 6	44 ± 1
3	15 ± 1	25 ± 1	18 ± 1	19 ± 1
4	1200 ± 30	1200 ± 20	1400 ± 40	1200 ± 20
5	4.7 ± 0.8	6 ± 1	6 ± 1	5 ± 1
6	0.80 ± 0.09	1.30 ± 0.07	1.20 ± 0.05	0.70 ± 0.04
7	11 ± 1	11 ± 4	10 ± 1	11 ± 2
8	3.8 ± 0.5	4.4 ± 0.7	3 ± 1	5 ± 1
9	4.3 ± 0,1	370 ± 98	8± 2	4.6 ± 0.5
10	4 ± 1	13 ± 1	6.5 ± 0.9	14± 2
11	5 ± 1	10 ± 1	10 ± 2	13 ± 1
12	130 ± 4	240 ± 23	242 ± 31	218 ± 13
13	838 ± 116	185 ± 8	304 ± 49	801 ± 30

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14	25 ± 9	42 ± 3	18 ± 1	46 ± 3
15	40 ± 3	13 ± 2	22 ± 1	42 ± 5
16	14 ± 4	10 ± 1	21 ± 9	65 ± 3
17	1.75 ± 0.07	1.20 ± 0.04	5 ± 2	1.7 ± 0.8
18	0.71 ± 0.08	0.9 ± 0.03	3 ± 1	3.0 ± 0.8
19	190 ± 6	200 ± 8	220 ± 3	220 ± 5
20	31 ± 6	24 ± 6	42 ± 7	32 ± 5
21	10 ± 1	20 ± 1	16 ± 3	10 ± 1.5
22	2600 ± 300	1800 ± 10	1700 ± 20	1500 ± 500
23	30 ± 6	14 ± 1	20 ± 5	92 ± 2
24	1.2 ± 0.2	1.5 ± 0.7	12 ± 3	1.7 ± 0.9
25	11.1 ± 0.2	17 ± 3	16 ± 4	9 ± 8
26	8 ± 2	10 ± 3	7 ± 2	7 ± 3
27	37 ± 3	89 ± 8	87 ± 16	98 ± 5

[a]IC_{50} values are expressed as the compound concentration (nM) that inhibits the cell growth by 50%. Data are the average (±SD) of three experiments.

The cytotoxicity measurements show that a high activity in the nM range is observed for all the derivatives, with the exception of compounds **4** (*o*-OMe) and **22** (*m,m*-diCF_3), the IC_{50} values of which fall in the μM range (**4**: IC_{50} = 1.2-1.4 μM).

Compounds with an *ortho* substituent in the phenyl ring are slightly more cytotoxic when bearing an electron withdrawing group, as in the cases of **3** (*o*-CF_3), **5** (*o*-F), **6** (*o*-Cl) or **7** (*o*-CN), than when having electron donor groups, like **2** (*o*-Me) or **4** (*o*-OMe). The remaining compounds display similar cytotoxicities, irrespective of their substitution pattern. Substrate **1**, with an unsubstituted phenyl ring, also exhibited a potent cytotoxic activity.

Compounds bearing two substituents on the phenyl ring also show IC_{50} values in the nM range. Like **6**, compounds **17** (*o*-Cl, *p*-F) and **18** (*o*-Cl, *p*-Me) display a chlorine atom in *ortho* position and show the lowest IC_{50} values of all the series. Thus, it may be concluded that the presence of a chlorine atom in *ortho* position increases the antitumoral activity of these derivatives. In contrast, the presence of two chlorine atoms in *ortho* position (compound **15,** *o,o*-diCl) slightly lowers the activity with respect to the previous substrates.

Compound **19** (*o*-Cl, *p*-CF_3), structurally related to **18** (*o*-Cl, *p*-Me), shows much less cytotoxic character (**19**; IC_{50}: HT-29, 190 nM; MCF-7, 200 nM; HeLa 220 nM). This decrease in activity is even greater in compound **22** (*m,m*-diCF_3), whose cytotoxicity falls in the μM range for all the assayed cell lines (**22**; IC_{50} = 1.7-2.6 μM).

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Compounds bearing two electron-donor groups in *ortho* position show a marked reduction in activity values, compounds **12** (*o*,*o*-diMe) and **13** (*o*,*o*-diEt) being representative examples. These compounds show IC_{50} values higher by a factor of over 100 than the remaining analogues, in agreement with the increase in cytotoxicity observed when electron-withdrawing groups are attached to the phenyl ring.

Not surprisingly, all these colchicine derivatives are also highly active against the non-tumor cell line HEK-293. However, it is worth mentioning that some of them have a considerable therapeutic window, as in compounds **14** (*o*,*o*-di-F), **16** (*o*-Me, *p*-F), **18** (*o*-Cl, *p*-Me) and **23** (*o*-diCl, *p*-F). These compounds display cytotoxic activities similar to those of the other derivatives, but they show a greater selectivity towards the non-tumor cell line, which makes them potentially interesting for further studies.

3.2. Effect on the apoptosis induction

The capacity of the synthetic compounds to induce apoptosis at dosis around their IC_{50} values was evaluated by measuring the translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the plasma membrane. Thus, HT-29 cells were incubated for 48 h in the presence of colchicine and the most representative compounds and then, the amount of annexin V was measured by flow cytometry.

Table 2 shows the percentage of viable, apoptotic and necrotic cells detected by flow cytometry after treatment with urea derivatives at dosis below their IC_{50} values. It can be observed that except for colchicine and compound **17**, more than a half of the population was still viable.

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Table 2. Apoptotic effect of colchicine and their derivatives.[a]

Compound	Conc. (nM)	Viable cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Control		82 ± 1	13 ± 3	2 ± 6
Colchicine	40	26 ± 1	73 ± 14	1 ± 1
Sunitinib	25000	75 ± 1	22 ± 14	3 ± 1
2 (*o*-MeC_6H_5)	25	64 ± 1	34 ± 7	2 ± 3
3 (*o*-$CF_3C_6H_5$)	20	96 ± 1	4 ± 3	1 ± 1
4 (*o*-$MeOC_6H_5$)	1000	55 ± 3	44 ± 3	1 ± 2
6 (*o*-ClC_6H_5)	0.5	58 ± 1	41 ± 2	1 ± 2
9 (*m*-ClC_6H_5)	5	62 ± 6	37 ± 8	1 ± 1
10 (*p*-$MeOC_6H_5$)	5	64 ± 5	35 ± 6	1 ± 2
11 (*p*-FC_6H_5)	5	51 ± 1	47 ± 1	2 ± 1
13 (*o,o*-diEtC_6H_5)	500	67 ± 1	32 ± 5	1 ± 1
14 (*o,o*-diFC_6H_5)	15	87 ± 1	12 ± 3	1 ± 2
16 (*o*-Me-*p*-FC_6H_5)	15	73 ± 3	26 ± 1	1 ± 4
17 (*o*-Cl-*p*-FC_6H_5)	5	46 ± 3	53 ± 1	1 ± 3
18 (*o*-Cl-*p*-MeC_6H_5)	2000	66 ± 3	33 ± 3	1 ± 1
22 (*m,m*-di$CF_3C_6H_5$)	20	96 ± 1	4 ± 1	1 ± 1
24 (*p*- $C_6H_5C_6H_5$)	1	86 ± 2	11 ± 1	3 ± 1

a Data are the average (±SD) of three experiments.

3.3. Effect on the expression of *hTERT*, c-*MYC* and *VEGF* genes

In order to determine the potential utility in cancer therapy of our derivatives, we began to investigate the effect they exert on the expression of the *hTERT, c-MYC* and *VEGF* genes on the HT-29 cell line. First, a conventional PCR was carried out as a preliminary study to qualitatively determine which compounds were able to modify the expression of these three genes in HT-29 cell line after 48 h of treatment (data not shown). This preliminary test let us select 10 urea derivatives and their minimal active concentration (see Figure 3) for a quantitative study of gene expression on the same cell line. As it is shown in Figure 3, the majority of these selected derivatives have one group on *ortho* position in the phenylurea subunit. In addition, the size of this *ortho* group is similar to that of a methyl group, except for the case of compound **14**, which bears two smaller F atoms in both *ortho* positions.

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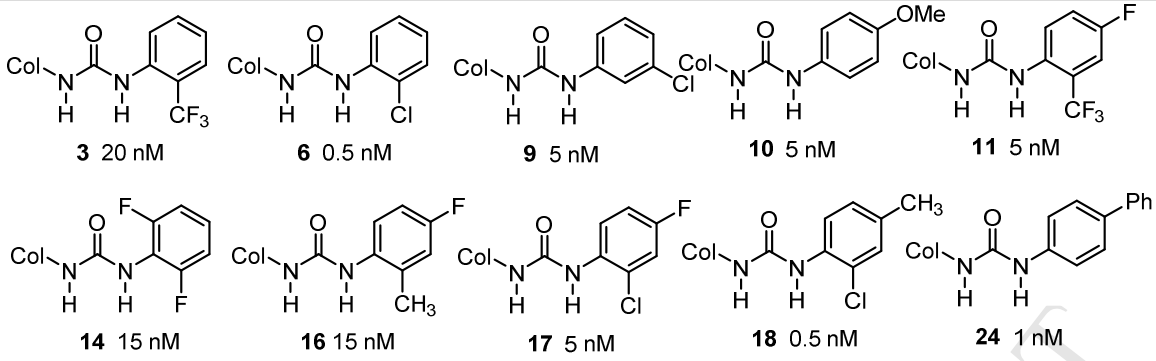

Figure 3. Selected compounds and concentrations for a quantitative gene expression test

The expression of the *c-MYC*, *hTERT* and *VEGF* genes on the HT-29 cell line, quantitatively determined by means of qPCR, is displayed in Table 3, in which the achieved values with colchicine and sunitinib are also displayed. All values were normalized to the number of viable cells that were present in each sample.

Table 3. Expression percentage for *c-MYC*, *hTERT* and *VEGF* genes[a]

Compound	Conc.	c-*Myc* gene exp.	*hTERT* gene exp.	*VEGF* gene exp.
Colchicine	25 nM	37 ± 3	87 ± 6	55 ± 2
Sunitinib	25 μM	47 ± 2	68 ± 2	46 ± 3
3	20 nM	107 ± 10	107 ± 13	101 ± 25
6	0.5 nM	25 ± 2	46 ± 5	30 ± 6
9	5 nM	104 ± 3	90 ± 8	95 ± 5
10	5 nM	55 ± 4	91 ± 4	70 ± 3
11	5 nM	52 ± 5	70 ± 3	59 ± 7
14	15 nM	88 ± 9	36 ± 5	38 ± 4
16	15 nM	65 ± 3	68 ± 2	63 ± 3
17	5 nM	58 ± 7	80 ± 8	64 ± 4
18	0.5 nM	85 ± 10	80 ± 5	70 ± 6
24	1 nM	85 ± 9	60 ± 3	72 ± 6

[a]Expression percentage of VEGF, hTERT and c-Myc genes after 48 h of incubation with DMSO (control), and selected compounds (at least three measurements were performed in each case). Gene expression was normalized using β-*ACTIN* as endogenous gene and the number of living cells in each assay.

The tested urea derivatives inhibit the expression of the studied genes to a considerable degree, even improving the results achieved by colchicine itself and sunitinib. Thus, colchicine was active at a concentration of 25 nM (see Table 3) but it has no activity when tested at lower concentrations

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(results not shown). Sunitinib was also quite active inhibiting the expression of the three oncogenes but at a dosis around 1000 times higher than colchicine.

As regards *c-MYC* gene expression, compound **6** (*o*-Cl) was the most active one and led to downregulation in the expression of this gene to 25% at 0.5 nM dosis (below its IC_{50}). *p*-Substituted phenylureas **10** (*p*-OMe), **11** (*o*-CF_3, *p*-F), **16** (*o*-Me, *p*-F) and **17** (*o*-Cl, *p*-F) performed also remarkably well, downregulating gene expression to 55, 52, 68 and 58 % respectively.

As regards *hTERT* gene, compounds **6** (*o*-Cl) and **14** (*o,o*-diF) are the most active ones, as they decrease gene expression to 46% and 36%, respectively. Compounds **11** (*o*-CF_3, *p*-F), **16** (*o*-Me, *p*-F) and **24** (*p*-Ph) are moderatively active with 70, 68 and 60 % of *hTERT* gene expression. In any case, all these analogues improve the values that display both colchicine (87%) and sunitinib (68%).

The same result was observed for *VEGF* gene expression, compounds **6** (*o*-Cl) and **14** (*o,o*-diF) being able to inhibit gene expression to 30% and 38% with respect to control. These two analogues improve the values that display both colchicine (55%) and sunitinib (46%). Furthermore, *p*-fluorophenylureas **11** (*o*-CF_3, *p*-F), **16** (*o*-Me, *p*-F) and **17** (*o*-Cl, *p*-F) also deserve mention, with *VEGF* expression values of 59%, 63% and 64%, respectively. The remainder of the assayed derivatives show a certain degree of inhibition, although to a lesser extent that the previous ones.

Compound **6** (*o*-Cl) was the most outstanding compound, and improves to a high extent the results obtained for colchicine and sunitinib. Indeed, it inhibits the three studied genes by an average of 34% at 0.5 nM dosis (below its IC_{50}).

3.4. Effect on c-MYC, VEGF and VEGFR-2 proteins

We next studied the action of colchicine derivatives on the c-MYC, VEGF and VEGFR-2 proteins. This study was performed by treating HT-29 cells with the selected compounds (see figure 3) for 48 hours. DMSO was used as a control, and colchicine and sunitinib as reference. Then, cell medium was collected to measure the percentage of secreted VEGF with respect to the control (untreated cells). On the other hand, cells were collected and lysated to measure the percentage of total c-MYC and VEGFR-2 proteins inside cell referring to control. All the assays were performed by ELISA (see methods and material section). The results are displayed in Table 4.

Table 4. Percentages of *c-MYC and VEGFR-2 proteins inside cells and secreted VEGF* [a]

Compound	Conc.	*c*-Myc protein	VEGFR-2 protein	VEGF secreted
Colchicine	25 nM	63 ± 4	94 ± 2	40 ± 2
Sunitinib	25 μM	100 ± 3	90 ± 9	45 ± 3

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3	20 nM	99 ± 3	100 ± 3	82 ± 5
6	0.5 nM	51 ± 3	38 ± 9	34 ± 3
9	5 nM	87 ± 4	15 ± 2	114± 11
10	5 nM	61 ± 6	95 ± 6	44 ± 2
11	5 nM	105 ± 8	63 ± 6	55 ± 8
14	15 nM	42 ± 3	35 ± 4	11 ± 2
16	15 nM	110 ± 3	62 ± 5	90 ± 4
17	5 nM	104 ± 8	15 ± 2	67 ± 5
18	0.5 nM	112 ± 5	45 ± 7	75 ± 6
24	1 nM	119 ± 3	74 ± 3	72 ± 4

[a] Expression percentage of total c-MYC and VEGFR-2 detected proteins and secreted VEGF to the medium after 48 h of incubation with DMSO (control) and selected compounds (at least three measurements were performed in each case).

As regards the effect on total c-MYC protein, the most active compounds were **6** (*o*-Cl), **14** (*o*-di-F) and colchicine itself, which caused reductions of the c-MYC protein to 51%, 42% and 63%, respectively, when compared to untreated cells.

Regarding the effect on total VEGFR-2 in cells, a significant reduction in the detected values for this protein is observed in HT-29 line with our set of derivatives. The most active derivatives were those bearing a chlorine atom in the phenyl ring as in the case of **9** (*m*-Cl) and **17** (*o*-Cl, *p*-F), which proved particularly active. Only 15% of VEGFR-2 protein was detected in both cases, in relation to untreated cells. Compounds **6** (*o*-Cl), **14** (*o*,*o*-diF) and **18** (*o*-Cl, *p*-CH_3), with 35, 38 and 45% of VEGFR-2 were also quite active in this biological target.

As regards the effect on VEGF protein secretion, compounds **6** and **14** were the most active ones, as they reduced the percentage of the secreted VEGF to 34% and a 11% respectively. Compound **10** (*p*-OMe), which exhibited similar activity to sunitinib and colchicine, was also quite active (around 40% of secreted protein, see Table 4).

4. Discussion of the results and conclusions

Except for **4** and **22**, all synthetized colchicine derivatives showed IC_{50} values in the nM range, with activities in some cases higher than those of the natural product. Some of them substantially improve the selectivity index against the studied tumoral cell lines, a point of interest for a possible further drug development. Compound **14** for instance exhibited lower IC_{50} values in tumor cell lines than in the non-tumor cell line HEK-293 (IC_{50}: HT-29, 25 nM; MCF-7, 42 nM; HeLa, 18 nM; HEK-293, 46 nM).

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Figure 4 represents the variation of *hTERT* gene expression, c-MYC detected protein and VEGF secreted protein in function of the selected derivatives. It can be observed that when either *hTERT* gene expression or when c-MYC protein disminishes around 40%, the secretion of VEGF protein to cell medium also decreases. For example, with compounds **6** (*o*-Cl) and **14** (*o*,*o*-diF), which are able to downregulate *hTERT* gene expression to 46 and 36%, respectively, and c-Myc protein to 51 and 42% respectively, secreted VEGF decreases to 34 and 11%, respectively. In the case of **10** (*p*-OMe), the reduction of c-MYC protein is sufficient to downregulate VEGF secretion to 44%, and with compound **24** (*p*-Ph), which downregulates to 60 % *hTERT* gene expression, secretion of VEGF protein goes down to 72 %. These results demonstrate that there are remarkable differences on the mode of action of these derivatives depending on the substitution pattern of the phenyl ring.

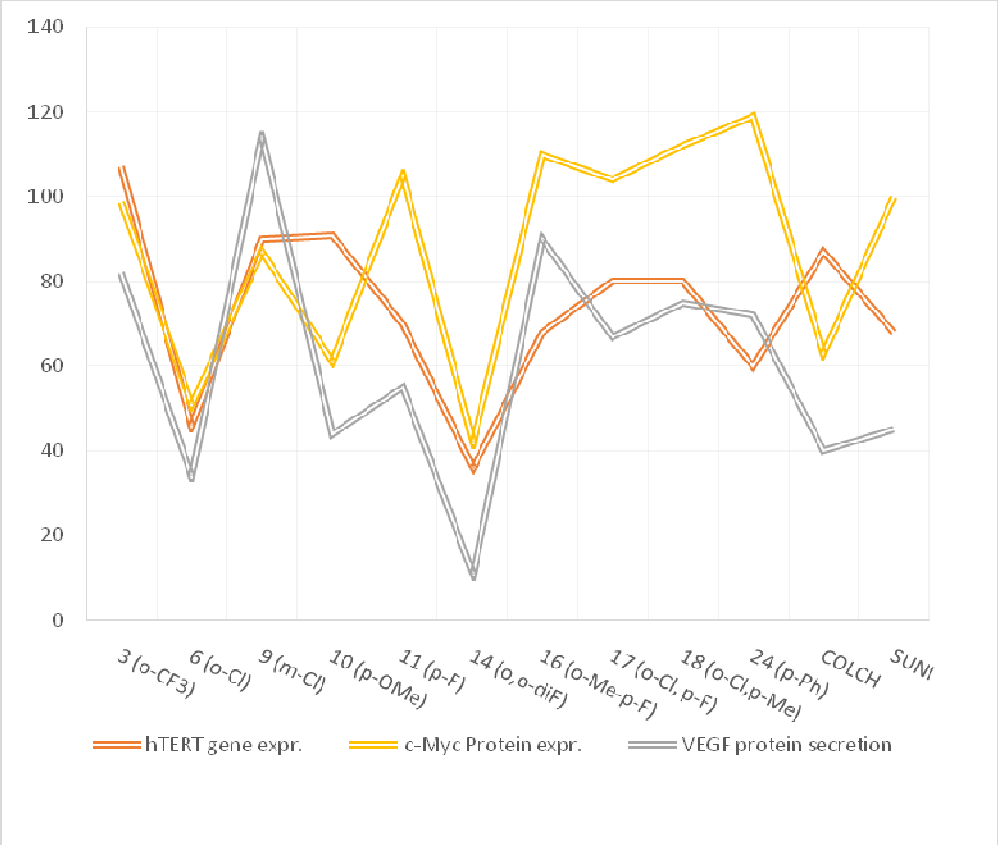

Figure 4. Variation of *hTERT* gene expression, c-MYC detected protein and VEGF secreted protein in function of the selected derivatives

Figure 5 represents the variation of the levels of *hTERT* gene expression, c-MYC protein and VEGFR-2 protein in function of the selected derivatives. It can be observed that only when the compound causes a reduction of both *hTERT* gene expression and c-MYC protein around 40%, the detection of VEGFR-2 decreases considerably. That is the case of compounds **6** (*o*-Cl) and **14** (*o*,*o*-diF), which are able to downregulate the *hTERT* gene expression to 46 and 36 %, the c-Myc protein to 51 and 42 % and the VEGFR-2 to 38 and 35 %, respectively. The cases of compounds **9** and **17**,

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which are not very active against *hTERT* gene or c-MYC protein but are able to decrease the detection of VEGFR-2 to 15%, are also worth mentioning. It may be conjectured that these compounds are downregulating this latter target through a mechanism different from that of the remaining derivatives.

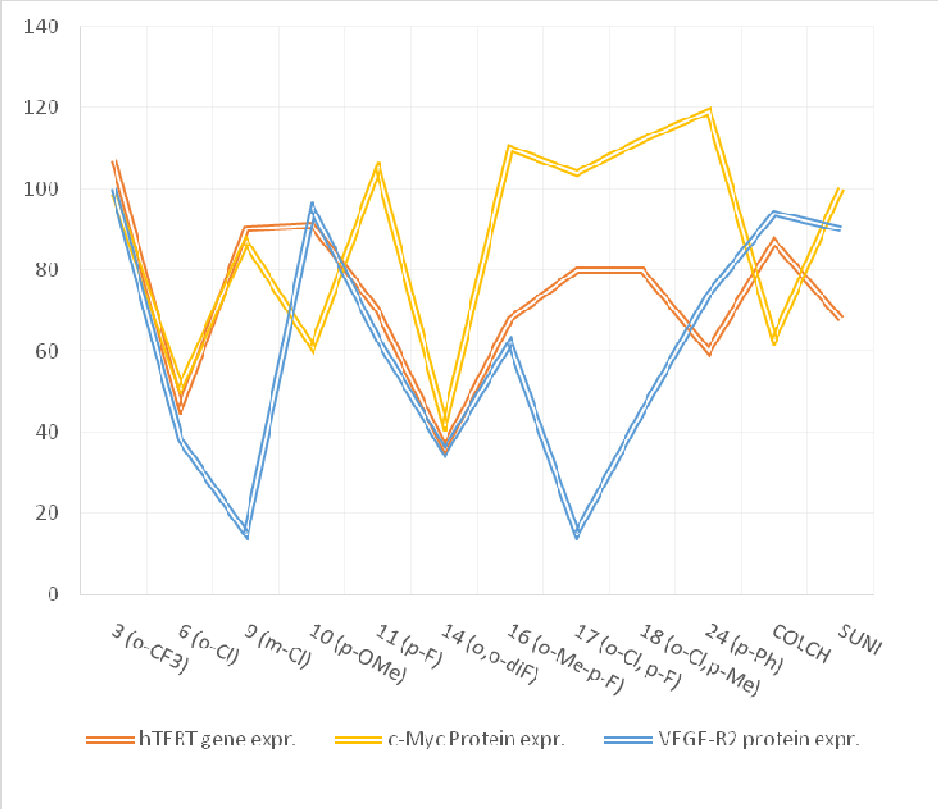

Figure 5. Variation of *hTERT* gene expression, c-MYC detected protein and detected VEGFR-2 protein in function of the selected derivatives

Figure 6 shows the results obtained on *hTERT*, *VEGF* and *c-MYC* genes, c-MYC and VEGFR-2 proteins and secretion of the VEGF protein to the cell medium.

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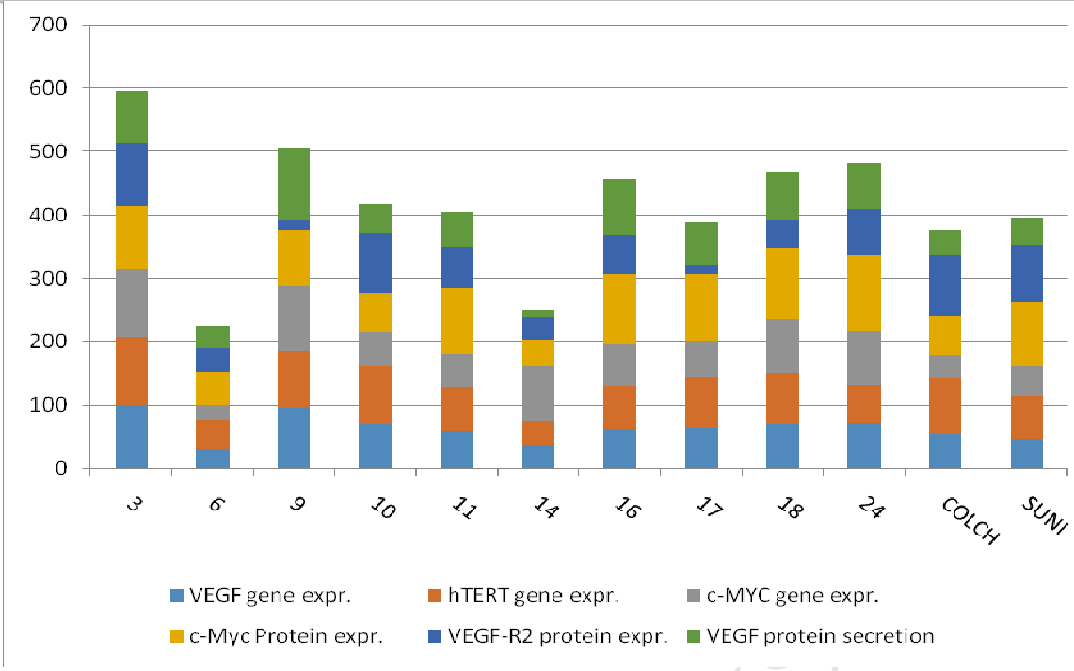

Figure 6. Summary of biological results of some urea derivatives and reference products

It can be observed that compounds **6** (*o*-Cl) and **14** (*o*,*o*-diF) were the most active ones on all the tested targets. Compound **6** exhibited similar IC_{50} values for all the tested cell lines (ranging 0.70-1.30 nM) while compound **14** exhibited lower IC_{50} values for tumoral cell lines than for HEK-293 (see Tables 1 and 3). It thus seems that compound **14** could be more selective than **6** towards tumoral cells and therefore less nocive for non-tumor cells. In any case, both derivatives are very active in downregulating all the tested targets at dosis 0.5 nM for **6** and 15 nM for **14**, which are below their IC_{50} values (see Table 5).

Table 5. Summary of the results obtained in the biological assays of the most active urea derivatives

Comp.	IC_{50} HT-29	IC_{50} HEK-293	c-*Myc* gen.	*hTERT* gen.	*VEGF* gen.	*c*-Myc protein	VEGFR-2 protein	VEGF secreted
6	0.80 nM	0.70 nM	25 %	46 %	30 %	51 %	38 %	34 %
14	25 nM	46 nM	88 %	36 %	38 %	42 %	35 %	11 %
Colch.	13 nM	13 nM	37 %	87 %	55 %	63 %	94 %	40 %

In summary, it can be concluded that compounds **6** and **14** may be good candidates for further in vivo assays against cancer. Thus, the arylurea unit enhances the potential of colchicine as an anticancer agent.

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5. Materials and methods

5.1. Chemical Procedures.

Conversion of deacetyl colchicine trifluoroacetate salt 28 into uretane 29. Compound **28** (472 mg, ca. 1 mmol) was dissolved under Ar in dry CH_2Cl_2 (5 mL), cooled in an ice bath and treated with a solution of phenyl chloroformiate (190 μL, 1.5 mmol) and pyridine (200 μL, 2.5 mmol) in dry CH_2Cl_2 (1 mL). The mixture was then stirred for 1 h at 0 ºC and then for 2 h at room temperature. Work-up (extraction with CH_2Cl_2) and column chromatography on silica gel (elution with hexane-EtOAc 1:4) afforded **29** (420 mg, 88%).

Conversion of 29 into urea derivatives 2, 9, 10, 12, 13 and 27. Uretane **29** (48 mg, 0.1 mmol) was dissolved under Ar in dry CH_2Cl_2 (0.5 mL) and treated with a solution of triethyl amine (21 μL, 0.15 mmol) and the appropriate amine (0.1 mmol) in dry CH_2Cl_2 (0.5 mL). The reaction mixture was then stirred for 24-96 h at room temperature (TLC monitoring). Work-up (extraction with CH_2Cl_2), was followed by column chromatography of the oily residue on silica gel (elution with EtOAc to EtOAc-MeOH 98:2) to afford the desired urea derivatives. For individual yields, see the synthetic Scheme.

Conversion of 28 into urea derivatives 1, 3-8, 11 and 14-26. The appropriate aromatic amine (1 mmol) was dissolved under Ar in dry CH_2Cl_2 (3 mL), cooled in an ice bath and treated with a solution of phenyl chloroformiate (190 μL, 1.5 mmol) and pyridine (200 μL, 2.5 mmol) in dry CH_2Cl_2 (1 mL). The mixture was then stirred for 2 h at room temperature and then worked up. The residue was subjected to column chromatography on silica gel (elution with hexane-EtOAc 9:1) to afford the corresponding urethane.

The urethane from above (1 mmol) and compound **28** (472 mg, 1 mmol) were dissolved under Ar in dry pyridine (3 mL) and heated at 80 ºC for 12-16 h (TLC monitoring). Work-up (extraction with CH_2Cl_2) was followed by column chromatography of the oily residue on silica gel (elution with EtOAc to EtOAc-MeOH 98:2) to afford the desired urea derivatives. For individual yields, see the synthetic Scheme.

(*S*)-1-Phenyl-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)urea (1): off-white solid, mp 198-199°C; $[\alpha]_D$ –44; ^{1}H NMR (300 MHz) δ 8.25 (1H, br s, NH), 8.00 (1H, br s, NH), 7.42 (1H, d, *J* = 10.8 Hz), 7.25 (2H, br d, *J* ~ 8 Hz), 7.12 (3H, br t, *J* ~ 7.5 Hz), 6.94 (1H, d, *J* = 10.8 Hz), 6.86 (1H, t, *J* = 7.2 Hz), 6.53 (1H, s), 4.80 (1H, m), 3.96 (6H, s), 3.90 (3H, s), 3.67 (3H, s), 2.50-2.35 (3H, br m), 1.85-1.75 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0, 164.1, 155.2, 154.6, 153.7, 151.3, 141.7, 139.5, 137.8, 134.6, 125.6 (C), 135.8, 131.8, 128.7 (x 2), 122.3, 119.7 (x 2), 113.5, 107.7, 52.4 (CH), 38.3, 30.4 (CH_2), 61.8, 61.5, 56.5, 56.2 (CH_3); IR ν_{max} 3330 br, 1698,

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1614, 1590, 1544, 1488, 1251, 1091 cm^{-1}; HR ESMS *m/z* 477.2018 ($M+H^+$). Calcd. for $C_{27}H_{29}N_2O_6$, 477.2025.

(*S*)-1-(1,2,3,10-Tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)-3-(*o*-tolyl)urea (2): off-white solid, mp 169-170 °C; $[\alpha]_D$ −15; ^{1}H NMR (300 MHz) δ 7.85 (1H, br s, NH), 7.50 (1H, dd, *J* = 8, 1.5 Hz), 7.37 (1H, d, *J* = 10.8 Hz), 7.30 (1H, br s, NH), 7.05-6.95 (3H, br m), 6.90-6.80 (2H, br m), 6.52 (1H, s), 4.67 (1H, m), 3.94 (3H, s), 3.92 (3H, s), 3.88 (3H, s), 3.65 (3H, s), 2.50-2.35 (3H, br m), 2.06 (3H, s), 1.80-1.70 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0, 164.1, 155.3, 154.3, 153.7, 151.3, 141.7, 137.4, 137.0, 134.5, 129.5, 125.6 (C), 135.8, 131.2, 130.4, 126.6, 123.8, 123.1, 113.3, 107.5, 52.8 (CH), 37.7, 30.3 (CH_2), 61.8, 61.5, 56.5, 56.2, 17.8 (CH_3); IR ν_{max} 3340 br, 1698, 1614, 1589, 1538, 1488, 1456, 1249, 1091 cm^{-1}; HR ESMS *m/z* 491.2167 ($M+H^+$). Calcd. for $C_{28}H_{31}N_2O_6$, 491.2182.

(*S*)-1-(1,2,3,10-Tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)-3-(2-trifluoromethyl-phenyl)urea (3): off-white solid, mp 203-204 °C; $[\alpha]_D$ −77; ^{1}H NMR (300 MHz) δ 7.90 (1H, br s, NH), 7.85 (1H, br d, *J* ~ 6 Hz, NH), 7.79 (1H, d, *J* = 8 Hz), 7.52 (1H, br s), 7.44 (1H, d, *J* = 10.8 Hz), 7.38 (1H, br d, *J* ~ 7.8 Hz), 7.22 (1H, br t, *J* ~ 7.8 Hz), 7.00-6.90 (2H, m), 6.53 (1H, s), 4.68 (1H, m), 4.00 (3H, s), 3.94 (3H, s), 3.90 (3H, s), 3.67 (3H, s), 2.50-2.35 (3H, br m), 1.85-1.75 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0 (C=O), 164.3, 154.7 (C=O), 154.2, 153.6, 151.3, 141.7, 137.3, 137.0 (center point of quadruplet with $^3J_{C-F}$ ~ 1.6 Hz), 134.5, 125.5, 124.0 (center point of quadruplet with $^1J_{C-F}$ ~ 273 Hz), 120.0 (center point of quadruplet with $^2J_{C-F}$ ~ 28.5 Hz) (C), 135.9, 132.4, 131.1, 125.6 (center point of quadruplet with $^3J_{C-F}$ ~ 5.5 Hz), 124.5, 122.5, 113.5, 107.4, 53.0 (CH), 37.4, 30.2 (CH_2), 61.7, 61.4, 56.5, 56.2 (CH_3); ^{19}F NMR (282 MHz) δ −61.5; IR ν_{max} 3300 br, 1704, 1616, 1583, 1544, 1456, 1322, 1249, 1093 cm^{-1}; HR ESMS *m/z* 545.1893 ($M+H^+$). Calcd. for $C_{28}H_{28}F_3N_2O_6$, 545.1899.

(*S*)-1-(2-Methoxyphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (4): off-white solid, mp 180-181 °C; $[\alpha]_D$ −28; ^{1}H NMR (300 MHz) δ 7.80 (1H, br s, NH), 7.76 (1H, dd, *J* = 7.8, 1.5 Hz), 7.35 (1H, d, *J* = 10.8 Hz), 7.31 (1H, d, *J* = 8 Hz), 6.90 (1H, d, *J* = 10.8 Hz), 6.80 (1H, td, *J* = 7.8, 1.5 Hz), 6.70 (1H, td, *J* = 7.8, 1.5 Hz), 6.64 (1H, dd, *J* = 7.8, 1.5 Hz), 6.52 (1H, br d, *J* ~ 6 Hz, NH), 6.50 (1H, s), 4.67 (1H, m), 4.01 (3H, s), 3.95 (3H, s), 3.90 (3H, s), 3.69 (3H, s), 3.57 (3H, s), 2.45-2.30 (3H, br m), 1.80-1.70 (1H, br m); ^{13}C NMR (75 MHz) δ 179.9, 164.2, 154.3, 153.7, 153.4, 151.3, 148.1, 141.8, 137.1, 134.6, 128.6, 125.7 (C), 135.5, 131.4, 122.1, 120.8, 119.2, 113.0, 110.0, 107.5, 52.8 (CH), 37.6, 30.2 (CH_2), 61.7, 61.5, 56.6, 56.3, 55.7 (CH_3); IR ν_{max} 3340 br, 1694, 1588, 1536, 1460, 1242, 1089 cm^{-1}; HR ESMS *m/z* 507.2129 ($M+H^+$). Calcd. for $C_{28}H_{31}N_2O_7$, 507.2131.

(*S*)-1-(2-Fluorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (5): off-white solid, mp 189-190 °C; $[\alpha]_D$ −71; ^{1}H NMR (300 MHz) δ 7.95 (1H, br s, NH), 7.87 (1H, br d, *J* ~ 1.5 Hz), 7.82 (1H, td, *J* = 8, 1.5 Hz), 7.55 (1H, br d, *J* ~ 3 Hz, NH), 7.40 (1H, d,

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J = 10.8 Hz), 6.95 (1H, d, *J* = 10.8 Hz), 6.85-6.75 (3H, br m), 6.47 (1H, s), 4.70 (1H, m), 4.00 (3H, s), 3.93 (3H, s), 3.89 (3H, s), 3.67 (3H, s), 2.40-2.30 (3H, br m), 1.85-1.75 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0, 164.3, 154.4, 154.2, 153.7, 152.5 (center point of doublet with $^{1}J_{C-F}$ ~ 242 Hz), 151.3, 141.7, 137.5, 134.6, 127.8 (doublet with $^{2}J_{C-F}$ ~ 10.2 Hz), 125.6 (C), 136.1, 131.3, 124.1 (center point of doublet with $^{3}J_{C-F}$ ~ 3.3 Hz), 122.2 (doublet with $^{3}J_{C-F}$ ~ 7.5 Hz), 121.3, 114.5 (doublet with $^{2}J_{C-F}$ ~ 19.5 Hz), 113.6, 107.5, 52.8 (CH), 37.7, 30.2 (CH_2), 61.8, 61.5, 56.6, 56.2 (CH_3); ^{19}F NMR (282 MHz) δ −131.3; IR ν_{max} 3320 br, 1704, 1620, 1588, 1544, 1249 cm^{-1}; HR ESMS *m/z* 495.1936 (M+H$^+$). Calcd. for $C_{27}H_{28}FN_2O_6$, 495.1931.

(*S*)-1-(2-Chlorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (6): off-white solid, mp 190-191 °C; $[\alpha]_D$ +3; ^{1}H NMR (300 MHz) δ 8.00 (1H, br s), 7.87 (1H, dd, *J* = 8, 1.5 Hz), 7.85 (1H, br d, *J* ~ 6 Hz, NH), 7.70 (1H, br s, NH), 7.42 (1H, d, *J* = 10.8 Hz), 7.03 (1H, dd, *J* = 8, 1.5 Hz), 6.96 (1H, d, *J* = 10.8 Hz), 6.95 (1H, td, *J* = 8, 1.5 Hz), 6.72 (1H, td, *J* = 8, 1.5 Hz), 6.45 (1H, s), 4.67 (1H, m), 4.03 (3H, s), 3.95 (3H, s), 3.89 (3H, s), 3.68 (3H, s), 2.40-2.30 (3H, br m), 1.85-1.80 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0, 164.3, 154.3, 154.2, 153.7, 151.3, 141.7, 137.5, 136.1, 134.5, 125.6 (C), 136.1, 131.2, 128.7, 127.2, 122.5, 121.0, 113.6, 107.4, 52.9 (CH), 37.4, 30.2 (CH_2), 61.8, 61.5, 56.7, 56.2 (CH_3); IR ν_{max} 3320 br, 1702, 1614, 1586, 1536, 1253 cm^{-1}; HR ESMS *m/z* 511.1635 (M+H$^+$). Calcd. for $C_{27}H_{28}{}^{35}ClN_2O_6$, 511.1636.

(*S*)-1-(2-Cyanophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (7): off-white solid, mp 177-178 °C; $[\alpha]_D$ −54; ^{1}H NMR (400 MHz) δ 8.03 (2H, br d, *J* ~ 8.5 Hz), 7.95 (1H, br s, NH), 7.80 (1H, br s, NH), 7.40 (2H, m), 7.25 (1H, br t, *J* ~ 8 Hz), 6.95 (1H, br d, *J* ~ 10.7 Hz), 6.89 (1H, br t, *J* ~ 7.5 Hz), 6.55 (1H, s), 4.70 (1H, m), 4.00 (3H, br s), 3.96 (3H, s), 3.93 (3H, s), 3.70 (3H, s), 2.55-2.35 (3H, br m), 1.95 (1H, br m); ^{13}C NMR (75 MHz) δ 180.1, 164.3, 153.8, 153.5, 153.2, 151.2, 142.6, 141.5, 137.2, 134.5, 125.6, 116.9, 100.4 (C), 135.9, 133.7, 132.5, 131.5, 121.6, 119.9, 113.5, 107.3, 52.9 (CH), 37.2, 30.1 (CH_2), 61.7, 61.4, 56.5, 56.1 (CH_3); IR ν_{max} 3300 br, 1704, 1586, 1534, 1249, 1231 cm^{-1}; HR ESMS *m/z* 502.1982 (M+H$^+$). Calcd. for $C_{28}H_{28}N_3O_6$, 502.1978.

(*S*)-1-(1,2,3,10-Tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)-3-(2-trifluoromethoxy-phenyl)urea (8): off-white solid, mp 186-187 °C; $[\alpha]_D$ −58; ^{1}H NMR (500 MHz) δ 7.95 (1H, br s), 7.91 (1H, dd, *J* = 8.3, 1.5 Hz), 7.70 (1H, br s, NH), 7.65 (1H, br d, *J* ~5.5 Hz, NH), 7.42 (1H, d, *J* = 10.8 Hz), 7.00 (1H, dt, *J* = 8.3, 1.5 Hz), 6.95 (1H, d, *J* = 10.8 Hz), 6.92 (1H, td, *J* = 8.3, 1.5 Hz), 6.77 (1H, td, *J* = 8.3, 1.5 Hz), 6.52 (1H, s), 4.70 (1H, m), 4.00 (3H, br s), 3.95 (3H, s), 3.91 (3H, s), 3.67 (3H, s), 2.50-2.35 (3H, br m), 1.80 (1H, m); ^{13}C NMR (125 MHz) δ 180.2, 164.4, 154.2, 154.1, 153.7, 151.3, 141.8, 137.8, 137.4, 134.5, 132.0, 125.7, 120.6 (center point of quadruplet with $^{1}J_{C-F}$ ~ 257 Hz) (C), 136.0, 131.1, 127.0, 121.7, 121.1, 119.8, 113.5, 107.4, 53.0 (CH), 37.5, 30.2 (CH_2), 61.7, 61.6, 56.6, 56.3 (CH_3); ^{19}F NMR (282 MHz) δ −58.2; IR ν_{max}

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3330 br, 1702, 1614, 1542, 1247 cm^{-1}; HR ESMS *m/z* 561.1834 ($M+H^+$). Calcd. for $C_{28}H_{28}F_3N_2O_7$, 561.1848.

(*S*)-1-(3-Chlorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (9): off-white solid, mp 179-180 °C; $[\alpha]_D$ −26; ^{1}H NMR (300 MHz) δ 8.60 (1H, br s, NH), 8.05 (1H, s), 7.50 (1H, d, *J* = 10.8 Hz), 7.35 (1H, t, *J* = 2 Hz), 7.30-7.20 (2H, m), 7.10-7.00 (2H, m), 6.85 (1H, dd, *J* = 8, 2 Hz), 6.56 (1H, s), 4.88 (1H, m), 4.02 (3H, br s), 3.97 (3H, s), 3.93 (3H, s), 3.71 (3H, s), 2.60-2.35 (3H, br m), 1.85 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0, 164.1, 155.0, 154.8, 153.9, 151.3, 141.8, 141.2, 138.2, 134.6, 134.2, 125.5 (C), 136.1, 132.0, 129.7, 121.9, 119.1, 117.1, 113.8, 107.7, 52.3 (CH), 38.7, 30.5 (CH_2), 61.9, 61.5, 56.6, 56.3 (CH_3); IR ν_{max} 3330 br, 1701, 1589, 1541, 1484, 1250 cm^{-1}; HR ESMS *m/z* 511.1622 ($M+H^+$). Calcd. for $C_{27}H_{28}{}^{35}ClN_2O_6$, 511.1636.

(*S*)-1-(4-Methoxyphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (10): off-white solid, mp 179-180 °C; $[\alpha]_D$ −13; ^{1}H NMR (300 MHz) δ 7.97 (1H, s), 7.85 (1H, br s, NH), 7.40 (1H, d, *J* = 10.8 Hz), 7.15 (2H, apparent d, *J* ~ 9 Hz), 6.95 (1H, br d, *J* ~ 8 Hz), 6.92 (1H, d, *J* = 10.8 Hz), 6.70 (2H, apparent d, *J* ~ 9 Hz), 6.55 (1H, s), 4.80 (1H, m), 3.95 (3H, s), 3.94 (3H, s), 3.90 (3H, s), 3.69 (3H, s), 3.67 (3H, s), 2.50 (1H, m), 2.45-2.35 (2H, br m), 1.80 (1H, br m); ^{13}C NMR (75 MHz) δ 179.9, 164.0, 155.7, 155.6, 154.5, 153.7, 151.3, 141.7, 137.7, 134.6, 132.2, 125.6 (C), 135.7, 131.7, 122.7 (x 2), 114.1 (x 2), 113.3, 107.6, 52.5 (CH), 38.2, 30.4 (CH_2), 61.8, 61.5, 56.5, 56.2, 55.5 (CH_3); IR ν_{max} 3320 br, 1689, 1613, 1588, 1544, 1510, 1225 cm^{-1}; HR ESMS *m/z* 507.2145 ($M+H^+$). Calcd. for $C_{28}H_{31}N_2O_7$, 507.2131.

(*S*)-1-(4-Fluorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (11): off-white solid, mp 177-178 °C; $[\alpha]_D$ −23; ^{1}H NMR (500 MHz) δ 8.20 (1H, br s, NH), 8.05 (1H, s), 7.45 (1H, d, *J* = 10.8 Hz), 7.18 (2H, dd, *J* = 9, 4.8 Hz), 7.10 (1H, br d, *J* ~ 8 Hz, NH), 6.96 (1H, d, *J* = 10.8 Hz), 6.82 (2H, t, *J* = 9 Hz), 6.55 (1H, s), 4.82 (1H, m), 3.96 (6H, s), 3.92 (3H, s), 3.69 (3H, s), 2.52 (1H, m), 2.45-2.35 (2H, br m), 1.85-1.80 (1H, br m); ^{13}C NMR (125 MHz) δ 180.0, 164.1, 158.6 (center point of doublet with $^1J_{C-F}$ ~ 240 Hz), 155.3, 154.8, 153.8, 151.3, 141.8, 138.0, 135.3, 134.6, 125.5 (C), 136.0, 131.7, 121.7 (x 2, doublet with $^3J_{C-F}$ ~ 7.5 Hz), 115.3 (x 2, doublet with $^2J_{C-F}$ ~ 22 Hz), 113.6, 107.6, 52.5 (CH), 38.4, 30.4 (CH_2), 61.9, 61.5, 56.5, 56.3 (CH_3); ^{19}F NMR (282 MHz) δ −121.6; IR ν_{max} 3320 br, 1698, 1614, 1588, 1544, 1508, 1249, 1089 cm^{-1}; HR ESMS *m/z* 495.1912 ($M+H^+$). Calcd. for $C_{27}H_{28}FN_2O_6$, 495.1931.

(*S*)-1-(2,6-Dimethylphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (12): off-white solid, mp 231-233 °C; $[\alpha]_D$ −20; ^{1}H NMR (400 MHz, 50 °C)† δ 7.30-7.25 (2H, m), 7.15 (3H, br m), 6.76 (1H, d, *J* = 10.8 Hz), 6.52 (1H, s), 5.80 (1H, br s, NH), 4.80 (1H, br s, NH), 4.65 (1H, m), 3.95 (6H, s), 3.91 (3H, s), 3.68 (3H, s), 2.50-2.35 (2H, br m), 2.32 (6H, br s), 2.20-2.10 (1H, m), 1.60-1.50 (1H, br m); ^{13}C NMR (125 MHz)‡ δ 179.6, 164.1, 155.7, 153.6, 151.5, 141.8, 134.4, 129.0 (br), 125.6 (C), 135.1, 131.1, 112.2, 107.5, 52.7 (CH), 37.8 (br), 30.3 (CH_2),

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61.8, 61.5, 56.3, 56.2, 18.5 (x 2) (CH_3); IR ν_{max} 3330 br, 1687, 1614, 1588, 1544, 1488, 1460, 1249, 1091 cm^{-1}; HR ESMS *m/z* 505.2359 ($M+H^+$). Calcd. for $C_{29}H_{33}N_2O_6$, 505.2338.

†Several ^1H signals were very broadened at room temperature due to hindered rotation.

‡A number of ^{13}C signals did not emerge from the background due to hindered rotation.

(*S*)-1-(2,6-Diethylphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (13): off-white solid, mp 245-247 °C; $[\alpha]_D$ –68; ^1H NMR (400 MHz, 50 °C)† δ 7.35-7.15 (5H, br m), 6.75 (1H, d, *J* = 10.7 Hz), 6.51 (1H, s), 5.70 (1H, br s, NH), 4.70-4.60 (2H, m), 3.96 (3H, s), 3.95 (3H, s), 3.91 (3H, s), 3.68 (3H, s), 2.70 (4H, br m), 2.50-2.30 (2H, br m), 2.10-2.00 (1H, m), 1.60-1.50 (1H, br m), 1.20 (6H, t, *J* = 7.5 Hz); ^{13}C NMR (125 MHz)‡ δ 179.6, 164.1, 156.3, 153.6, 151.4, 141.8, 134.4, 132.8 (br), 125.8 (C), 135.0, 131.1 (br), 112.2 (br), 107.5, 52.7 (CH), 37.8 (br), 30.3, 24.9 (x 2) (CH_2), 61.6, 61.5, 56.3, 56.2, 15.0 (x 2) (CH_3); IR ν_{max} 3340 br, 1687, 1614, 1588, 1547, 1247, 1091 cm^{-1}; HR ESMS *m/z* 533.2667 ($M+H^+$). Calcd. for $C_{31}H_{37}N_2O_6$, 533.2651.

†Several ^1H signals were very broadened at room temperature due to hindered rotation.

‡A number of ^{13}C signals did not emerge from the background due to hindered rotation.

(*S*)-1-(2,6-Difluorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (14): off-white solid, mp 190-192 °C; $[\alpha]_D$ –36; ^1H NMR (500 MHz) δ 8.25 (1H, br s, NH), 8.15 (1H, s), 7.40 (1H, d, *J* = 10.8 Hz), 7.30 (1H, br d, *J* ~ 8.8 Hz, NH), 7.05 (1H, m), 6.87 (1H, d, *J* = 10.8 Hz), 6.85 (2H, t, *J* = 8 Hz), 6.55 (1H, s), 4.93 (1H, m), 3.93 (3H, s), 3.91 (3H, s), 3.72 (3H, s), 3.64 (3H, s), 2.55-2.40 (3H, br m), 1.95-1.85 (1H, br m); ^{13}C NMR (125 MHz) δ 180.0, 163.8, 158.3 (x 2, center point of double doublet with $^1J_{C-F}$ ~ 248 Hz and $^3J_{C-F}$ ~ 5 Hz), 154.9, 154.0, 153.7, 151.3, 141.7, 137.9, 134.7, 125.6, 116.1 (triplet with $^2J_{C-F}$ ~ 16 Hz) (C), 135.5, 132.1, 125.9 (triplet with $^3J_{C-F}$ ~ 9 Hz), 113.3, 111.5 (x 2, double doublet with $^2J_{C-F}$ ~ 19 Hz and $^4J_{C-F}$ ~ 5 Hz), 107.7, 52.6 (CH), 39.0, 30.5 (CH_2), 61.8, 61.4, 56.3, 56.2 (CH_3); ^{19}F NMR (282 MHz) δ −119.1; IR ν_{max} 3300 br, 1704, 1614, 1588, 1536, 1462, 1244, 1091 cm^{-1}; HR ESMS *m/z* 513.1828 ($M+H^+$). Calcd. for $C_{27}H_{27}F_2N_2O_6$, 513.1837.

(*S*)-1-(2,6-Dichlorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (15): off-white solid, mp 176-178 °C; $[\alpha]_D$ –82; ^1H NMR (500 MHz) δ 8.25 (1H, br s, NH), 8.17 (1H, br s), 7.37 (1H, d, *J* = 10.8 Hz), 7.28 (2H, d, *J* = 8 Hz), 7.05 (1H, t, *J* = 8 Hz), 6.97 (1H, br d, *J* ~ 8 Hz, NH), 6.82 (2H, d, *J* = 10.8 Hz), 6.55 (1H, s), 4.87 (1H, m), 3.91 (3H, s), 3.90 (3H, s), 3.65 (3H, br s), 3.62 (3H, s), 2.55-2.40 (3H, br m), 1.95-1.90 (1H, br m); ^{13}C NMR (125 MHz) δ 180.0, 163.7, 154.8, 154.0, 153.7, 151.3, 141.7, 137.7, 134.8 (x 2), 134.7, 134.0, 125.6 (C), 135.3, 132.0, 128.3 (x 2), 127.6, 113.0, 107.7, 52.5 (CH), 38.9, 30.5 (CH_2), 61.8, 61.5, 56.2, 56.1 (CH_3); IR ν_{max} 3320 br, 1698, 1657, 1614, 1580, 1547, 1460, 1432, 1249, 1091 cm^{-1}; HR ESMS *m/z* 545.1229 ($M+H^+$). Calcd. for $C_{27}H_{27}{}^{35}Cl_2N_2O_6$, 545.1246.

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(*S*)-1-(4-Fluoro-2-methylphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)urea (16): off-white solid, mp 182-184 °C; $[\alpha]_D$ −6; ^{1}H NMR (500 MHz) δ 7.95 (1H, br s, NH), 7.45-7.35 (3H, br m), 7.20 (1H, br d, *J* ~ 7 Hz, NH), 6.94 (1H, d, *J* = 11 Hz), 6.75-6.65 (2H, br m), 6.54 (1H, s), 4.70 (1H, m), 3.95 (6H, s), 3.91 (3H, s), 3.66 (3H, s), 2.55-2.35 (3H, br m), 2.05 (3H, s), 1.80-1.70 (1H, br m); ^{13}C NMR (125 MHz) δ 180.0, 164.2, 159.4 (center point of doublet with $^1J_{C-F}$ ~ 243 Hz), 155.5, 154.7, 153.8, 151.3, 141.8, 137.7, 134.5, 132.8 (doublet with $^4J_{C-F}$ ~ 3 Hz), 132.5 (doublet with $^3J_{C-F}$ ~ 8 Hz), 125.6 (C), 136.1, 131.2, 125.1 (doublet with $^3J_{C-F}$ ~ 8 Hz), 116.6 (doublet with $^2J_{C-F}$ ~ 22 Hz), 113.6, 113.0 (doublet with $^2J_{C-F}$ ~ 22 Hz), 107.5, 53.0 (CH), 37.8, 30.3 (CH_2), 61.8, 61.5, 56.5, 56.3, 18.0 (CH_3); ^{19}F NMR (282 MHz) δ −120.2; IR ν_{max} 3320 br, 1698, 1614, 1588, 1544, 1490, 1249, 1091 cm^{-1}; HR ESMS *m/z* 509.2083 (M+H$^+$). Calcd. for $C_{28}H_{30}FN_2O_6$, 509.2088.

(*S*)-1-(2-Chloro-4-fluorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)urea (17): off-white solid, mp 181-183 °C; $[\alpha]_D$ +17; ^{1}H NMR (300 MHz) δ 8.00 (1H, s), 7.95 (1H, br d, *J* ~ 6.5 Hz, NH), 7.80 (1H, dd, *J* = 9.2, 5.7 Hz), 7.70 (1H, br s, NH), 7.44 (1H, d, *J* = 10.8 Hz), 6.98 (1H, d, *J* = 10.8 Hz), 6.77 (1H, dd, *J* = 8.2, 3 Hz), 6.67 (1H, ddd, *J* = 9.2, 8.2, 3 Hz), 6.51 (1H, s), 4.66 (1H, m), 4.03 (3H, s), 3.95 (3H, s), 3.90 (3H, s), 3.67 (3H, s), 2.50-2.30 (3H, br m), 1.90-1.80 (1H, br m); ^{13}C NMR (75 MHz) δ 180.0, 164.3, 157.3 (center point of doublet with $^1J_{C-F}$ ~ 243 Hz), 154.6, 154.4, 153.8, 151.3, 141.7, 137.7, 134.5, 132.6 (doublet with $^4J_{C-F}$ ~ 3 Hz), 125.5, 123.0 (doublet with $^3J_{C-F}$ ~ 10.5 Hz) (C), 136.3, 131.2, 122.1 (doublet with $^3J_{C-F}$ ~ 7.5 Hz), 115.6 (doublet with $^2J_{C-F}$ ~ 25 Hz), 113.9 (doublet with $^2J_{C-F}$ ~ 22 Hz), 113.8, 107.4, 53.0 (CH), 37.4, 30.1 (CH_2), 61.8, 61.5, 56.7, 56.2 (CH_3); ^{19}F NMR (282 MHz) δ −120.6; IR ν_{max} 3300 br, 1700, 1614, 1588, 1531, 1486, 1251 cm^{-1}; HR ESMS *m/z* 529.1529 (M+H$^+$). Calcd. for $C_{27}H_{27}{}^{35}ClFN_2O_6$, 529.1541.

(*S*)-1-(2-Chloro-4-methylphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (18): off-white solid, mp 194-196 °C; $[\alpha]_D$ −33; ^{1}H NMR (300 MHz) δ 7.95 (1H, br s, NH), 7.70 (1H, d, *J* =8.3 Hz), 7.50 (2H, m), 7.40 (1H, d, *J* = 10.8 Hz), 6.94 (1H, d, *J* = 10.8 Hz), 6.88 (1H, br d, *J* ~ 2 Hz), 6.77 (1H, br dd, *J* ~ 8.3, 2 Hz), 6.50 (1H, s), 4.67 (1H, m), 4.00 (3H, s), 3.95 (3H, s), 3.90 (3H, br s), 3.68 (3H, s), 2.45-2.30 (3H, br m), 2.15 (3H, s), 1.85-1.80 (1H, m); ^{13}C NMR (75 MHz) δ 180.0, 164.2, 154.4, 154.1, 153.7, 151.3, 141.7, 137.4, 134.6, 133.5, 132.5, 125.6, 122.7 (C), 135.9, 131.3, 129.1, 127.9, 121.4, 113.5, 107.5, 53.0 (CH), 37.5, 30.2 (CH_2), 61.9, 61.5, 56.6, 56.2 (CH_3); IR ν_{max} 3300 br, 1704, 1687, 1562, 1544, 1253, 1095 cm^{-1}; HR ESMS *m/z* 525.1789 (M+H$^+$). Calcd. for $C_{28}H_{30}{}^{35}ClN_2O_6$, 525.1792.

(*S*)-1-(2-Chloro-4-trifluoromethylphenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a] heptalen-7-yl) urea (19): off-white solid, mp 174-176 °C; $[\alpha]_D$ −10; ^{1}H NMR (500 MHz) δ 8.35 (1H, br s, NH), 8.03 (1H, br s), 7.95 (1H, br d, *J* ~ 8.8 Hz), 7.90 (1H, br s, NH), 7.48 (1H, d, *J* = 10.8 Hz), 7.25 (1H, s), 7.04 (1H, d, *J* = 10.7 Hz), 7.02 (1H, dd, *J* = 8.8, 2 Hz), 6.52

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(1H, s), 4.66 (1H, m), 4.06 (3H, br s), 3.96 (3H, s), 3.90 (3H, s), 3.69 (3H, s), 2.50-2.35 (3H, br m), 1.90-1.85 (1H, m); ^{13}C NMR (75 MHz) δ 180.2, 164.3, 154.6, 153.9, 153.6, 151.3, 141.8, 139.3, 137.9, 134.4, 125.4, 123.8 (quadruplet with $^{2}J_{C-F}$ ~ 33 Hz), 123.6 (center point of quadruplet with $^{1}J_{C-F}$ ~ 268 Hz), 121.2 (C), 136.6, 131.1, 125.7 (quadruplet with $^{3}J_{C-F}$ ~ 3.5 Hz), 124.3 (quadruplet with $^{3}J_{C-F}$ ~ 3.5 Hz), 119.2, 114.2, 107.4, 53.1 (CH), 37.1, 30.1 (CH_2), 61.8, 61.5, 56.8, 56.2 (CH_3); ^{19}F NMR (282 MHz) δ −62.6; IR ν_{max} 3300 br, 1709, 1614, 1588, 1531, 1322, 1251 cm^{-1}; HR ESMS *m/z* 579.1489 (M+H$^+$). Calcd. for $C_{28}H_{27}{}^{35}ClF_3N_2O_6$, 579.1509.

(*S*)-1-(2,4-Dichlorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (20): off-white solid, mp 178-180 °C; $[\alpha]_D$ +25; ^{1}H NMR (500 MHz) δ 8.10 (1H, br s, NH), 7.98 (1H, br s), 7.83 (1H, d, *J* = 8.8 Hz), 7.70 (1H, br s, NH), 7.45 (1H, d, *J* = 10.8 Hz), 7.05-6.95 (2H, m), 6.86 (1H, dd, *J* = 8.8, 2 Hz), 6.50 (1H, s), 4.65 (1H, m), 4.04 (3H, br s), 3.95 (3H, s), 3.90 (3H, s), 3.66 (3H, s), 2.50-2.30 (3H, br m), 1.85-1.75 (1H, m); ^{13}C NMR (125 MHz) δ 180.1, 164.3, 154.6, 153.9, 153.8, 151.3, 141.7, 137.8, 135.0, 134.5, 126.5, 125.5, 122.3 (C), 136.4, 131.2, 128.2, 127.2, 121.0, 114.0, 107.5, 53.0 (CH), 37.2, 30.2 (CH_2), 61.8, 61.5, 56.7, 56.3 (CH_3); IR ν_{max} 3300 br, 1704, 1614, 1581, 1544, 1519, 1249 cm^{-1}; HR ESMS *m/z* 545.1217 (M+H$^+$). Calcd. for $C_{27}H_{27}{}^{35}Cl_2N_2O_6$, 545.1246.

(*S*)-1-(3,5-Difluorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (21): off-white solid, mp 204-206 °C; $[\alpha]_D$ −24; ^{1}H NMR (500 MHz) δ 8.80 (1H, br s, NH), 8.02 (1H, br s), 7.51 (1H, d, *J* = 10.8 Hz), 7.25 (1H, br s, NH), 7.05 (1H, d, *J* = 10.8 Hz), 6.95 (2H, br d, $^{3}J_{HF}$ ~ 9 Hz), 6.56 (1H, s), 6.30 (1H, br t, $^{3}J_{HF}$ ~ 9 Hz), 4.87 (1H, m), 4.05 (3H, s), 3.97 (3H, s), 3.91 (3H, s), 3.72 (3H, s), 2.55-2.35 (3H, br m), 1.90-1.80 (1H, br m); ^{13}C NMR (125 MHz) δ 180.0, 164.2, 163.2 (x 2, center point of double doublet with $^{1}J_{C-F}$ ~ 242 and $^{3}J_{C-F}$ ~ 15 Hz), 155.0, 154.4, 153.9, 151.3, 142.3 (triplet with $^{3}J_{C-F}$ ~ 13 Hz), 141.8, 138.3, 134.5, 125.5 (C), 136.3, 131.8, 114.1, 107.7, 101.4 (x 2, double doublet with $^{2}J_{C-F}$ ~ 22 and $^{4}J_{C-F}$ ~ 7 Hz), 96.8 (triplet with $^{2}J_{C-F}$ ~ 25 Hz), 52.2 (CH), 38.6, 30.4 (CH_2), 61.8, 61.5, 56.5, 56.2 (CH_3); ^{19}F NMR (282 MHz) δ −111.1; IR ν_{max} 3320 br, 1704, 1613, 1544, 1249 cm^{-1}; HR ESMS *m/z* 513.1820 (M+H$^+$). Calcd. for $C_{27}H_{27}F_2N_2O_6$, 513.1837.

(*S*)-1-[3,5-Bis(trifluoromethyl)phenyl]-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a] heptalen-7-yl)urea (22): off-white solid, mp 205-207 °C; $[\alpha]_D$ −31; ^{1}H NMR (500 MHz) δ 9.25 (1H, br s, NH), 8.10 (1H, s), 7.85 (2H, s), 7.56 (1H, d, *J* = 10.8 Hz), 7.35 (2H, m), 7.08 (1H, d, *J* = 10.8 Hz), 6.58 (1H, s), 4.96 (1H, m), 4.02 (3H, br s), 3.99 (3H, s), 3.93 (3H, s), 3.75 (3H, s), 2.55-2.40 (3H, br m), 1.95-1.90 (1H, br m); ^{13}C NMR (125 MHz) δ 180.0, 164.1, 155.2, 154.5, 154.1, 151.4, 142.0, 141.7, 138.5, 134.5, 131.8 (x 2, center point of quadruplet with $^{2}J_{C-F}$ ~ 33 Hz), 125.4, 123.5 (x 2, center point of quadruplet with $^{1}J_{C-F}$ ~ 270 Hz) (C), 136.6, 132.1, 118.1 (x 2, quadruplet with $^{3}J_{C-F}$ ~ 4 Hz), 114.8 (septuplet with $^{3}J_{C-F}$ ~ 3.5 Hz), 114.3, 107.7, 52.2 (CH), 38.9, 30.5 (CH_2), 61.9, 61.5, 56.6, 56.3 (CH_3); ^{19}F NMR (282 MHz) δ −63.4; IR ν_{max} 3320

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br, 1704, 1616, 1586, 1547, 1387, 1251, 1125, 1093 cm^{-1}; HR ESMS *m/z* 613.1751 ($M+H^+$). Calcd. for $C_{29}H_{27}F_6N_2O_6$, 613.1773.

(*S*)-1-(2,6-Dichloro-4-fluorophenyl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a] heptalen-7-yl) urea (23): off-white solid, mp 187-189 °C; $[\alpha]_D$ −72; ^{1}H NMR (500 MHz) δ 8.35 (1H, br s, NH), 8.21 (1H, br s), 7.40 (1H, d, *J* = 10.7 Hz), 7.15 (1H, br d, *J* ~ 8 Hz), 7.05 (2H, d, $^3J_{HF}$ = 8 Hz), 6.84 (1H, d, *J* = 10.7 Hz), 6.55 (1H, s), 4.87 (1H, m), 3.91 (3H, s), 3.90 (3H, s), 3.64 (3H, s), 3.62 (3H, s), 2.55-2.40 (3H, br m), 1.95-1.90 (1H, m); ^{13}C NMR (125 MHz) δ 180.0, 163.6, 159.8 (center point of doublet with $^1J_{C-F}$ ~ 250 Hz), 155.1, 154.3, 153.7, 151.3, 141.7, 138.0, 135.6 (x 2, doublet with $^3J_{C-F}$ ~ 12 Hz), 134.7, 130.7 (doublet with $^4J_{C-F}$ ~ 4 Hz), 125.5 (C), 135.4, 132.0, 115.8 (x 2, doublet with $^2J_{C-F}$ ~ 25 Hz), 113.1, 107.7, 52.5 (CH), 39.0, 30.5 (CH_2), 61.8, 61.5, 56.3, 56.1 (CH_3); ^{19}F NMR (282 MHz) δ −114.5; IR ν_{max} 3300 br, 1698, 1581, 1544, 1462, 1249, 1091 cm^{-1}; HR ESMS *m/z* 563.1151 ($M+H^+$). Calcd. for $C_{27}H_{26}{}^{35}Cl_2FN_2O_6$, 563.1152.

(*S*)-1-([1,1'-Biphenyl]-4-yl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl) urea (24): off-white solid, mp 197-199 °C; $[\alpha]_D$ +25; ^{1}H NMR (300 MHz) δ 8.25 (1H, br s, NH), 8.00 (1H, br s), 7.45-7.35 (3H, m), 7.30-7.15 (8H, br m), 6.92 (1H, d, *J* = 11 Hz), 6.47 (1H, s), 4.65 (1H, m), 3.93 (3H, br s), 3.89 (3H, s), 3.83 (3H, s), 3.65 (3H, s), 2.45-2.30 (3H, br m), 1.85-1.75 (1H, m); ^{13}C NMR (75 MHz+) δ 180.0, 164.1, 155.1, 154.7, 153.7, 151.3, 141.7, 141.0, 138.8, 137.9, 135.0, 134.6, 125.5 (C), 136.0, 131.6, 128.6 (x 2), 127.3 (x 2), 126.7 (x 2), 126.6, 120.0 (x 2), 113.7, 107.5, 52.5 (CH), 38.2, 30.4 (CH_2), 61.9, 61.5, 56.5, 56.2 (CH_3); IR ν_{max} 3300 br, 1700, 1613, 1590, 1544, 1536, 1488, 1251, 1093 cm^{-1}; HR ESMS *m/z* 553.2335 ($M+H^+$). Calcd. for $C_{33}H_{33}N_2O_6$, 553.2338.

(*S*)-1-(Naphthalen-1-yl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)urea (25): off-white solid, mp 180-182 °C; $[\alpha]_D$ −1; ^{1}H NMR (500 MHz) δ 8.25 (1H, br s, NH), 8.10 (1H, br s), 8.00 (1H, br s), 7.75 (1H, d, *J* = 8 Hz), 7.60 (1H, d, *J* = 7.5 Hz), 7.57 (1H, d, *J* = 8 Hz), 7.39 (1H, d, *J* = 10.8 Hz), 7.40-7.25 (3H, br m), 7.05 (1H, br s, NH), 6.80 (1H, d, *J* = 10.8 Hz), 6.55 (1H, s), 4.88 (1H, m), 3.92 (3H, br s), 3.90 (3H, s), 3.65 (3H, s), 3.57 (3H, s), 2.55-2.35 (3H, br m), 1.85-1.75 (1H, m); ^{13}C NMR (125 MHz) δ 180.0, 164.1, 156.0, 154.3, 153.7, 151.3, 141.7, 137.6, 134.6, 134.3, 134.0, 128.7, 125.6 (C), 135.7, 131.8, 128.2, 126.0, 125.9, 125.8, 125.0, 122.3, 121.7, 113.3, 107.6, 52.8 (CH), 38.5, 30.4 (CH_2), 61.8, 61.5, 56.3, 56.2 (CH_3); IR ν_{max} 3300 br, 1698, 1544, 1536, 1249 cm^{-1}; HR ESMS *m/z* 527.2176 ($M+H^+$). Calcd. for $C_{31}H_{31}N_2O_6$, 527.2182.

(*S*)-1-(Naphthalen-2-yl)-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)urea (26): off-white solid, mp 182-184 °C; $[\alpha]_D$ −38; ^{1}H NMR (400 MHz) δ 8.50 (1H, br s, NH), 8.15 (1H, br s), 7.85 (1H, br s), 7.56 (2H, br t, *J* ~ 8.5 Hz), 7.50 (1H, m), 7.46 (1H, d, *J* = 10.8 Hz), 7.30-7.20 (4H, br m), 6.96 (1H, d, *J* = 10.8 Hz), 6.53 (1H, s), 4.90 (1H, m), 3.96 (6H, s), 3.90 (3H, s), 3.74 (3H, s), 2.50-2.35 (3H, br m), 1.90-1.80 (1H, m); ^{13}C NMR (75 MHz) δ 180.0, 164.1,

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155.3, 154.6, 153.7, 151.3, 141.7, 137.9, 137.1, 134.6, 134.2, 129.7, 125.5 (C), 135.9, 131.8, 128.3, 127.4, 127.3, 125.9, 123.8, 120.5, 115.3, 113.6, 107.5, 52.4 (CH), 38.3, 30.4 (CH_2), 61.8, 61.5, 56.5, 56.2 (CH_3); IR ν_{max} 3300 br, 1698, 1544, 1249 cm^{-1}; HR ESMS *m/z* 527.2179 ($M+H^+$). Calcd. for $C_{31}H_{31}N_2O_6$, 527.2182.

(*S*)-1-Cyclohexyl-3-(1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl)urea (27): off-white solid, mp 168-170 °C; $[\alpha]_D$ –54; ^{1}H NMR (500 MHz) δ 7.70 (1H, s), 7.35 (1H, d, *J* = 10.7 Hz), 6.87 (1H, d, *J* = 10.7 Hz), 6.52 (1H, s), 5.95 (1H, br d, *J* ~ 7 Hz, NH), 5.00 (1H, br d, *J* ~ 8 Hz, NH), 4.60 (1H, m), 4.00 (3H, s), 3.93 (3H, s), 3.90 (3H, s), 3.66 (3H, s), 3.45 (1H, m), 2.48 (1H, m), 2.40-2.25 (2H, br m), 1.90-1.50 (6H, br m), 1.30-1.20 (2H, m), 1.10-0.95 (3H, br m); ^{13}C NMR (125 MHz) δ 180.0, 164.1, 156.5, 153.9, 153.6, 151.3, 141.7, 137.1, 134.6, 125.7 (C), 135.4, 131.3, 112.9, 107.5, 52.7, 48.8 (CH), 37.9, 34.1, 33.9, 30.3, 25.7, 25.1, 25.0 (CH_2), 61.8, 61.5, 56.5, 56.3 (CH_3); IR ν_{max} 3320 br, 1650. 1614, 1547, 1488, 1249, 1093 cm^{-1}; HR ESMS *m/z* 483.2486 ($M+H^+$). Calcd. for $C_{27}H_{35}N_2O_6$, 483.2495.

5.2. Biological studies. Materials and methods

5.2.1. Cell culture

Cell culture media were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, U.K.). Supplements and other chemicals not listed in this section were obtained from Sigma Chemical Co. (St. Louis, MO). Plastics for cell culture were supplied by Thermo Scientific BioLite. All tested compounds were dissolved in DMSO at a concentration of 20 μM and stored at -20°C until use.

HT-29, MCF-7, HeLa and HEK-293 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphotericin B (1.25 μg/mL), supplemented with 10% FBS.

5.2.2 MTT assay

A total of 5×10^3 HT-29, MCF-7, HeLa or HEK-293 cells in a total volume of 100 μL of their respective growth media were incubated with serial dilutions of the tested compounds. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) dye reduction assay in 96-well microplates was used, as previously described [26]. After 2 days of incubation (37 °C, 5% CO_2 in a humid atmosphere), 10 μL of MTT (5 mg/mL in phosphate-buffered saline, PBS) was added to each well, and the plate was incubated for a further 3 h (37 °C). The supernatant was discarded and replaced by 100 μL of DMSO to dissolve formazan crystals. The absorbance was then read at 490 nm by spectrophotometry. For all concentrations of compound, cell viability was expressed as the percentage of the ratio between the mean absorbance of treated cells and the mean

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absorbance of untreated cells. Three independent experiments were performed, and the IC_{50} values (i.e., concentration half inhibiting cell proliferation) were graphically determined.

5.2.3 Apoptosis assay

Apoptosis was determined by quantifying FITC-Annexin V translocation by means of flow cytometry. HT-29 cells were incubated with compounds for 48 h and then stained following instructions of BD Apoptosis DetectionTM Kit. Analysis was performed with a BD AccuriTM C6 flow cytometer.

5.2.4. RT-qPCR Assay

HT-29 cells at 70–80% confluence were collected and 1.5 x 10^5 cells were placed in a six well plate in 1.5 mL of medium. After 24h, cells were incubated with the corresponding compounds for 48 h. Cells were collected and the total cellular RNA from HT-29 cells was isolated using Ambion RNA extraction Kit according to the manufacturer's instructions. The cDNA was synthesized by MMLV-RT with 1–21 μg of extracted RNA and oligo(dT)15 according to the manufacturer's instructions.

Genes were amplified by use of a thermal cycler and StepOnePlus ™ Taqman ® probes. TaqMan ® Gene Expression Master Mix Fast containing the appropriate buffer for the amplification conditions, dNTPs, thermostable DNA polymerase enzyme and a passive reference probe was used. To amplify each of the genes the predesigned primers were used and sold by Life Technologies TaqMan ® Gene Expression Assays, Hs99999903-m1 (β-actin), Hs00972646-m1 (hTERT), Hs00153408-m1 (c-Myc) and Hs009900055-m1 (VEGF-A).

5.2.5. Detection of total c-Myc and VEGFR-2 proteins by ELISA

HT-29 cells at 70–80% confluence were collected and 1.5 x 10^5 cells were placed in a six well plate in 1.5 mL of medium. After 24h, cells were incubated with the corresponding compounds for 48 h. Cells were collected, washed twice in PBS and then cells were lysated with 200 μL of lysis buffer. Buffer we used for the cell lysis consists of 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton™ X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate. Immediately before use, we added PMSF (0.3 M stock in DMSO) to 1 mM and 50 μL/mL protease inhibitor cocktail (e.g., Sigma Cat. No. P-2714). Protein concentrations of the cell lysates were determined by Bradford method and then we carried out to a concentration of 200 μg/mL. Afterwards, we performed ELISA test according to the manufacturer's instructions. For determining total c-Myc, we used Invitrogen™ Novex™ c-Myc

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(Total) Human ELISA Kit de ThermoFisher® and total VEGFR-2 we used Sigma Human VEGFR-2 ELISA test (ref. RAB0328).

5.2.6. Detection of secreted VEGF by ELISA

HT-29 cells at 70–80% confluence were collected and 1.5 x 10^5 cells were placed in a six well plate in 1.5 mL of medium. After 24h, cells were incubated with the corresponding compounds for 48 h and then, cell culture media were collected. Protein concentrations of the cell media were determined by Bradford method and then we carried out to a concentration of 200 μg/mL. Afterwards, we performed ELISA test according to the manufacturer's instructions. We used Invitrogen™ Novex™ VEGF Human ELISA Kit de ThermoFisher.

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Supplementary Information

Additional figures, graphics, experimental data, physical, IR, NMR and mass spectrometry data of all new synthetic compounds are provided in the Supplementary Information.

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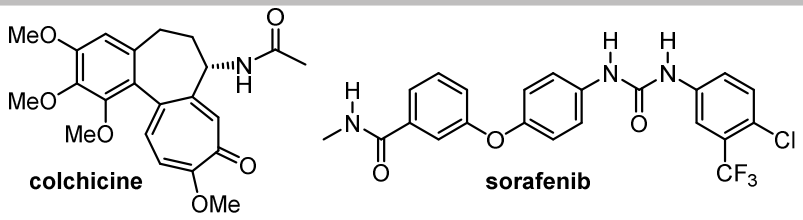

Figure 1

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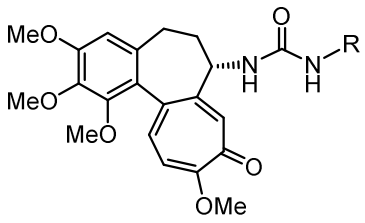

1 R = C_6H_5
2 R = *o*-MeC_6H_5
3 R = *o*-$CF_3C_6H_5$
4 R = *o*-$OMeC_6H_5$
5 R = *o*-FC_6H_5
6 R = *o*-ClC_6H_5
7 R = *o*-CNC_6H_5
8 R = *o*-$CF_3OC_6H_5$
9 R = *m*-ClC_6H_5
10 R = *p*-$MeOC_6H_5$
11 R = *p*-FC_6H_5
12 R = *o,o*-diMeC_6H_4
13 R = *o,o*-diEtC_6H_4
14 R = *o,o*-diFC_6H_4
15 R = *o,o*-diClC_6H_4
16 R = *o*-Me-*p*-FC_6H_4
17 R = *o*-Cl-*p*-FC_6H_4
18 R = *o*-Cl-*p*-MeC_6H_4
19 R = *o*-Cl-*p*-$CF_3C_6H_4$
20 R = *o,p*-diClC_6H_4
21 R = *m,m*-diFC_6H_4
22 R = *m,m*-di$CF_3C_6H_4$
23 R = *o,o*-diCl-*p*-FC_6H_3
24 R = *p*-$C_6H_5C_6H_5$
25 R = 1-naphthyl
26 R = 2-naphthyl
27 R = cyclohexyl

Figure 2

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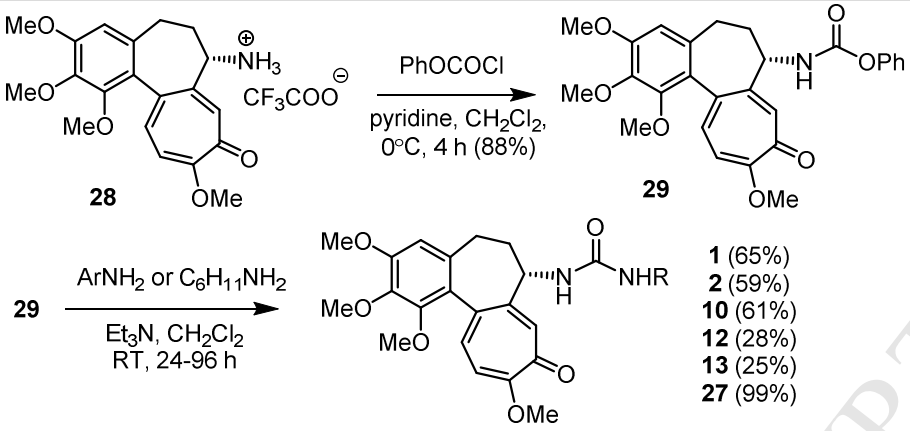

Scheme 1

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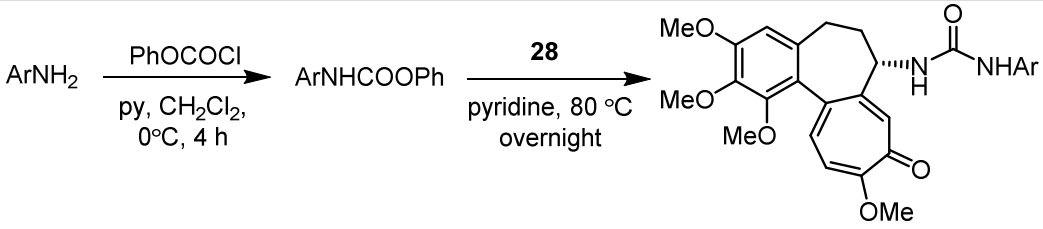

3 (16%)	**11** (38%)	**20** (38%)
4 (41%)	**14** (46%)	**21** (38%)
5 (53%)	**15** (27%)	**22** (22%)
6 (69%)	**16** (47%)	**23** (32%)
7 (60%)	**17** (55%)	**24** (31%)
8 (15%)	**18** (88%)	**25** (32%)
9 (32%)	**19** (47%)	**26** (27%)

Scheme 2

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Table 1.[a]

Compound	HT-29	MCF-7	HeLa	HEK-293
Colchicine	13 ± 2	20 ± 2	14 ± 1	13 ± 3
1	11 ± 2	12 ± 3	12 ± 2	10 ± 5
2	26 ± 2	119 ± 8	32 ± 6	44 ± 1
3	15 ± 1	25 ± 1	18 ± 1	19 ± 1
4	1200 ± 30	1200 ± 20	1400 ± 40	1200 ± 20
5	4.7 ± 0.8	6 ± 1	6 ± 1	5 ± 1
6	0.80 ± 0.09	1.30 ± 0.07	1.20 ± 0.05	0.70 ± 0.04
7	11 ± 1	11 ± 4	10 ± 1	11 ± 2
8	3.8 ± 0.5	4.4 ± 0.7	3 ± 1	5 ± 1
9	4.3 ± 0,1	370 ± 98	8± 2	4.6 ± 0.5
10	4 ± 1	13 ± 1	6.5 ± 0.9	14± 2
11	5 ± 1	10 ± 1	10 ± 2	13 ± 1
12	130 ± 4	240 ± 23	242 ± 31	218 ± 13
13	838 ± 116	185 ± 8	304 ± 49	801 ± 30
14	25 ± 9	42 ± 3	18 ± 1	46 ± 3
15	40 ± 3	13 ± 2	22 ± 1	42 ± 5
16	14 ± 4	10 ± 1	21 ± 9	65 ± 3
17	1.75 ± 0.07	1.20 ± 0.04	5 ± 2	1.7 ± 0.8
18	0.71 ± 0.08	0.9 ± 0.03	3 ± 1	3.0 ± 0.8
19	190 ± 6	200 ± 8	220 ± 3	220 ± 5
20	31 ± 6	24 ± 6	42 ± 7	32 ± 5
21	10 ± 1	20 ± 1	16 ± 3	10 ± 1.5
22	2600 ± 300	1800 ± 10	1700 ± 20	1500 ± 500
23	30 ± 6	14 ± 1	20 ± 5	92 ± 2
24	1.2 ± 0.2	1.5 ± 0.7	12 ± 3	1.7 ± 0.9
25	11.1 ± 0.2	17 ± 3	16 ± 4	9 ± 8
26	8 ± 2	10 ± 3	7 ± 2	7 ± 3
27	37 ± 3	89 ± 8	87 ± 16	98 ± 5

[a] *IC_{50} values are expressed as the compound concentration (nM) that inhibits the cell growth by 50%. Data are the average (±SD) of three experiments*

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Table 2.[a]

Compound	Conc. (nM)	Viable cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Control		82 ± 1	13 ± 3	2 ± 6
Colchicine	40	26 ± 1	73 ± 14	1 ± 1
Sunitinib	25000	75 ± 1	22 ± 14	3 ± 1
2 (*o*-MeC_6H_5)	25	64 ± 1	34 ± 7	2 ± 3
3 (*o*-$CF_3C_6H_5$)	20	96 ± 1	4 ± 3	1 ± 1
4 (*o*-$MeOC_6H_5$)	1000	55 ± 3	44 ± 3	1 ± 2
6 (*o*-ClC_6H_5)	0.5	58 ± 1	41 ± 2	1 ± 2
9 (*m*-ClC_6H_5)	5	62 ± 6	37 ± 8	1 ± 1
10 (*p*-$MeOC_6H_5$)	5	64 ± 5	35 ± 6	1 ± 2
11 (*p*-FC_6H_5)	5	51 ± 1	47 ± 1	2 ± 1
13 (*o*,*o*-diEtC_6H_5)	500	67 ± 1	32 ± 5	1 ± 1
14 (*o*,*o*-diFC_6H_5)	15	87 ± 1	12 ± 3	1 ± 2
16 (*o*-Me-*p*-FC_6H_5)	15	73 ± 3	26 ± 1	1 ± 4
17 (*o*-Cl-*p*-FC_6H_5)	5	46 ± 3	53 ± 1	1 ± 3
18 (*o*-Cl-*p*-MeC_6H_5)	2000	66 ± 3	33 ± 3	1 ± 1
22 (*m*,*m*-di$CF_3C_6H_5$)	20	96 ± 1	4 ± 1	1 ± 1
24 (*p*- $C_6H_5C_6H_5$)	1	86 ± 2	11 ± 1	3 ± 1

a Data are the average (±SD) of three experiments.

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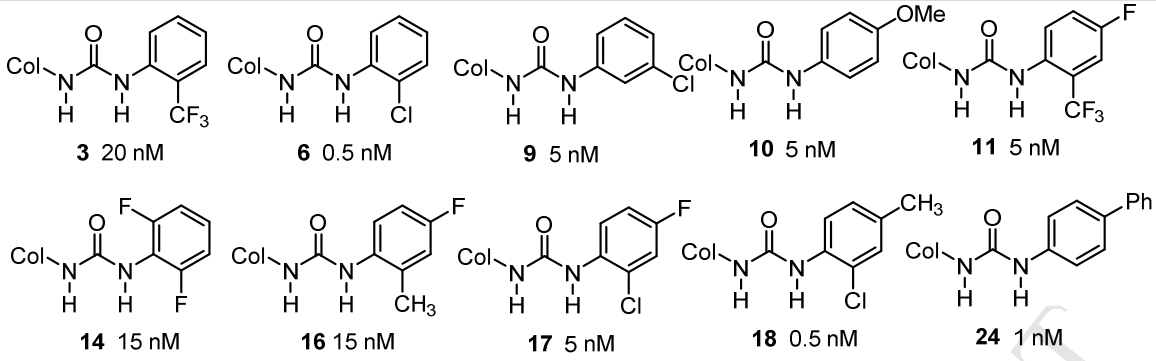

Figure 3.

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Table 3.

Compound	Conc.	c-*Myc* gene exp.	*hTERT* gene exp.	*VEGF* gene exp.
3	20 nM	107 ± 10	107 ± 13	101 ± 25
6	0.5 nM	25 ± 2	46 ± 5	30 ± 6
9	5 nM	104 ± 3	90 ± 8	95 ± 5
10	5 nM	55 ± 4	91 ± 4	70 ± 3
11	5 nM	52 ± 5	70 ± 3	59 ± 7
14	15 nM	88 ± 9	36 ± 5	38 ± 4
16	15 nM	65 ± 3	68 ± 2	63 ± 3
17	5 nM	58 ± 7	80 ± 8	64 ± 4
18	0.5 nM	85 ± 10	80 ± 5	70 ± 6
24	1 nM	85 ± 9	60 ± 3	72 ± 6
Colchicine	25 nM	37 ± 3	87 ± 6	55 ± 2
Sunitinib	25 μM	47 ± 2	68 ± 2	46 ± 3

[a]Expression percentage of VEGF, hTERT and c-Myc genes after 48 h of incubation with DMSO (control), and selected compounds (at least three measurements were performed in each case). Gene expression was normalized using β-*ACTIN* as endogenous gene.

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Table 4. [a]

Compound	Conc.	*c*-Myc protein	VEGFR-2 protein	VEGF secreted
3	20 nM	99 ± 3	100 ± 3	82 ± 5
6	0.5 nM	51 ± 3	38 ± 9	34 ± 3
9	5 nM	87 ± 4	15 ± 2	114± 11
10	5 nM	61 ± 6	95 ± 6	44 ± 2
11	5 nM	105 ± 8	63 ± 6	55 ± 8
14	15 nM	42 ± 3	35 ± 4	11 ± 2
16	15 nM	110 ± 3	62 ± 5	90 ± 4
17	5 nM	104 ± 8	15 ± 2	67 ± 5
18	0.5 nM	112 ± 5	45 ± 7	75 ± 6
24	1 nM	119 ± 3	74 ± 3	72 ± 4
Colchicine	25 nM	63 ± 4	94 ± 2	40 ± 2
Sunitinib	25 μM	100 ± 3	90 ± 9	45 ± 3

[a] Expression percentage of total c-MYC and VEGFR-2 detected proteins and secreted VEGF to the medium after 48 h of incubation with DMSO (control) and selected compounds (at least three measurements were performed in each case).

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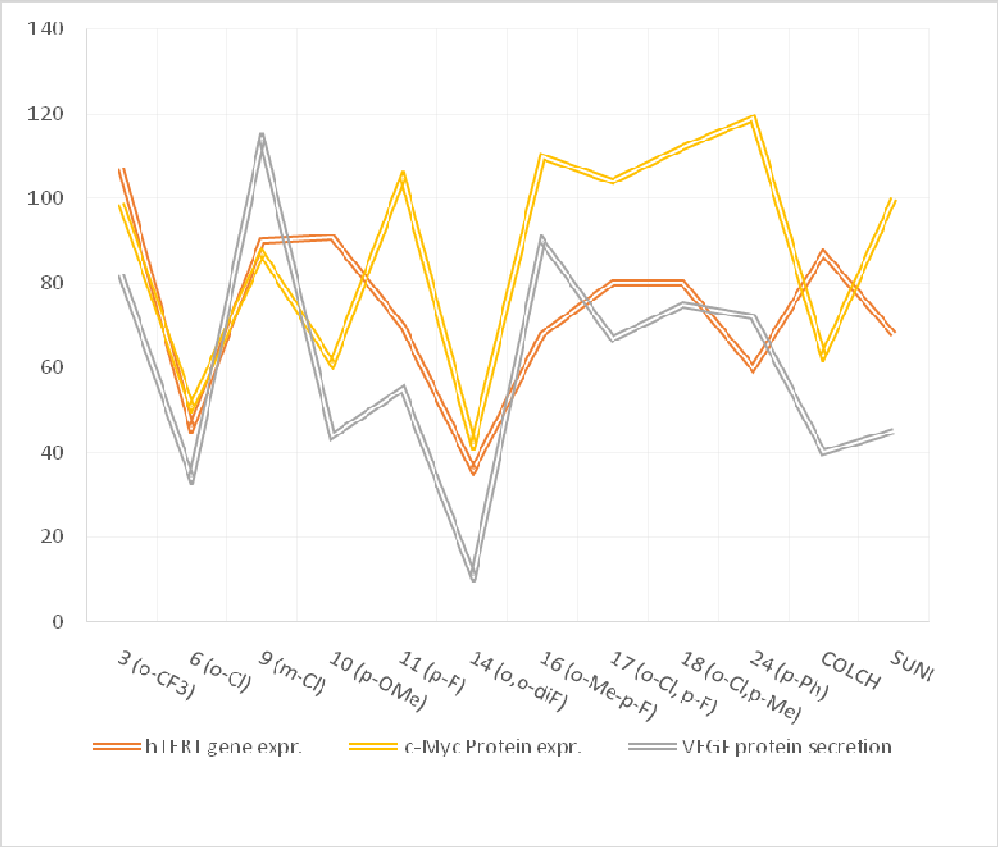

Figure 4.

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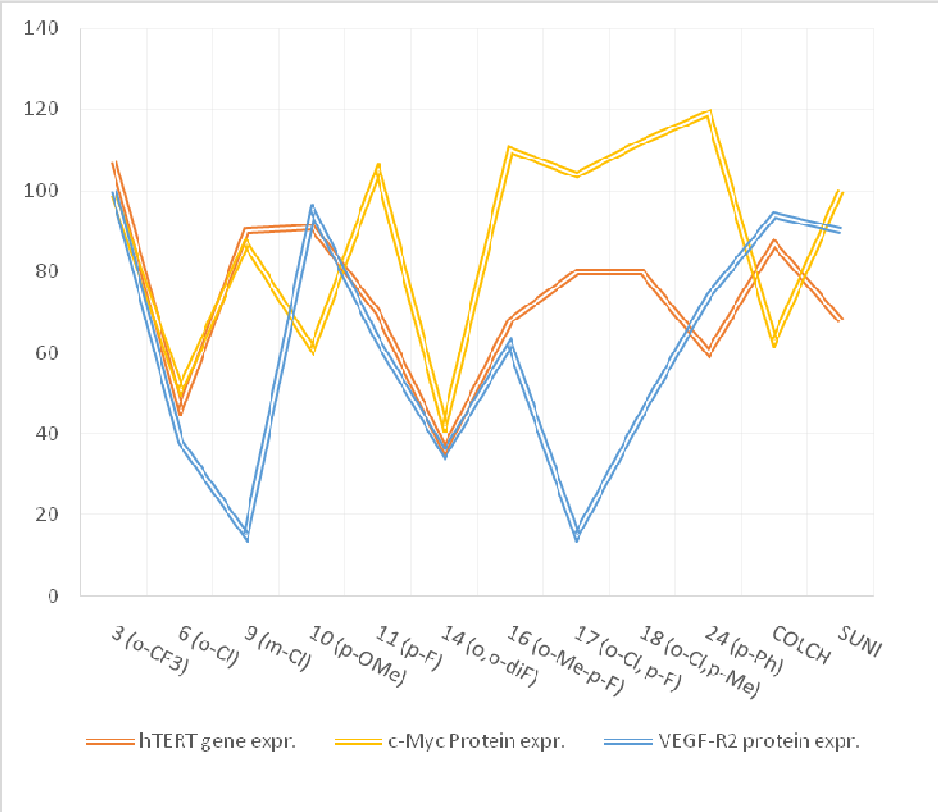

Figure 5.

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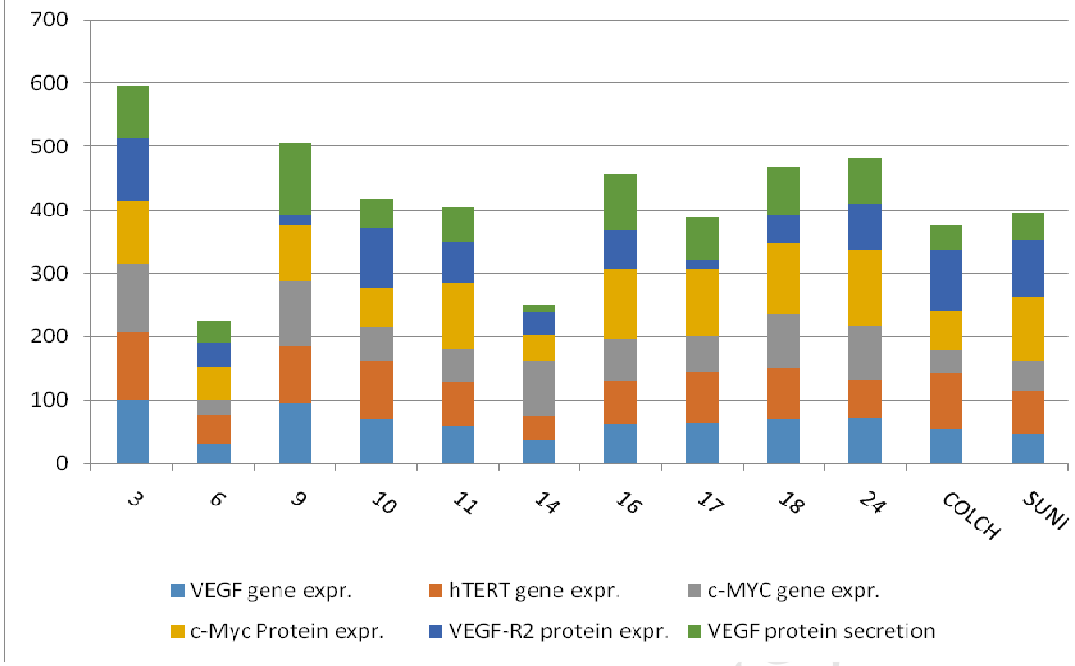

Figure 6.

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Table 5.

Comp.	IC_{50} HT-29	IC_{50} HEK-293	c-*Myc* gen.	*hTERT* gen.	*VEGF* gen.	*c*-Myc protein	VEGFR-2 protein	VEGF secreted
6	0.80 nM	0.70 nM	25 %	46 %	30 %	51 %	38 %	34 %
14	25 nM	46 nM	52 %	70 %	59 %	42 %	35 %	11 %
Colch.	13 nM	13 nM	37 %	87 %	55 %	63 %	94 %	40 %

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Arylureas derived from colchicine: Enhancement of colchicine oncogene downregulation activity

Víctor Blasco, Ana C. Cuñat, Juan F. Sanz-Cervera, J. Alberto Marco, Eva Falomir, Juan Murga and Miguel Carda

Highlights

- Twenty-seven ureas with a colchicine part and an aryl fragment have been synthesized.
- The arylurea unit causes a great improvement in anticancer properties.
- N-*o*,*o*-difluorophenylurea showed lower IC_{50} values for tumor cell lines than for HEK-293.
- N-*o*-Chlorophenylurea and N-*o*,*o*-difluorophenylurea were the most active agents.
- These two ureas downregulate the targets at a concentration below their IC_{50} values.

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