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Carbonic anhydrase inhibitors. Biphenylsulfonamides with inhibitory action towards the transmembrane, tumor-associated isozymes IX possess cytotoxic activity against human colon, lung and breast cancer cell lines

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Abstract

Reaction of 4,4-biphenyl-disulfonyl chloride with aromatic/heterocyclic sulfonamides also incorporating a free amino group, such as 4-aminobenzenesulfonamide, 4-aminoethyl-benzenesulfonamide, 6-chloro-4-aminobenzene-1,3-disulfonamide or 5-amino-1,3,4-thiadiazole-2-sulfonamide afforded bis-sulfonamides which have been tested as inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4..2.1.1). The compounds were rather modest inhibitors of isozymes CA I and XII, but were more efficient as inhibitors of the cytosolic CA II and transmembrane, tumor-associated CA IX (inhibition constants in the range of 21–129 nM gainst hCA II, and 23–79 nM against hCA IX, respectively). The new bis-sulfonamides also showed inhibition of growth of several tumor cell lines (*ex vivo*), with GI_{50} values in the range of 0.74–10.0 μg/mL against the human colon cancer cell line HCT116, the human lung cancer cell line H460 and the human breast cancer cell line MCF-7.

Keywords: *Carbonic anhydrase, CA-IX, inhibitors, cytotoxic, cancer cell lines, HCT 116, H460, MCF-7*

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in bacteria, archaea and eukaryotes, catalyzing a critically important physiologic reaction, hydration of carbon dioxide to bicarbonate and protons [1–4]. These enzymes are inhibited by several classes of compounds, such as sulfonamides [1,5–9], sulfamates [1,2] and sulfamides [1,2], some of which have pharmacologic applications for the treatment of glaucoma [5] obesity [6], cancer [8–12], epilepsy [7] and other neurological disorders [1,2] or as diuretics [5]. Bacterial, fungal and protozoan CAs belonging to the α-, β-, γ- and/or δ-CA gene families, which are present in many pathogens, started also to be considered recently as potential targets for the development of inhibitors with therapeutic applications [13–18]. Inhibitors belonging to the chemical classes mentioned above bind to the catalytic zinc ion within the enzyme cavity, as shown by means of X-ray crystallographic studies for many representatives, mainly in complex with the ubiquitous human isoform II (hCA II) [5,19–24]. A number of such derivatives are clinically used drugs, such as acetazolamide **1**, methazolamide **2**, ethoxzolamide **3**, dichlorophenamide **4**, dorzolamide **5**, brinzolamide **6**, etc., among others [1,25]. Other compounds are in clinical development as antitumor agents, such as indisulam **7** and COUMATE-667 **8** [1]. CA inhibitors (CAIs) are mainly used in therapy as diuretics and antiglaucoma agents but some of them

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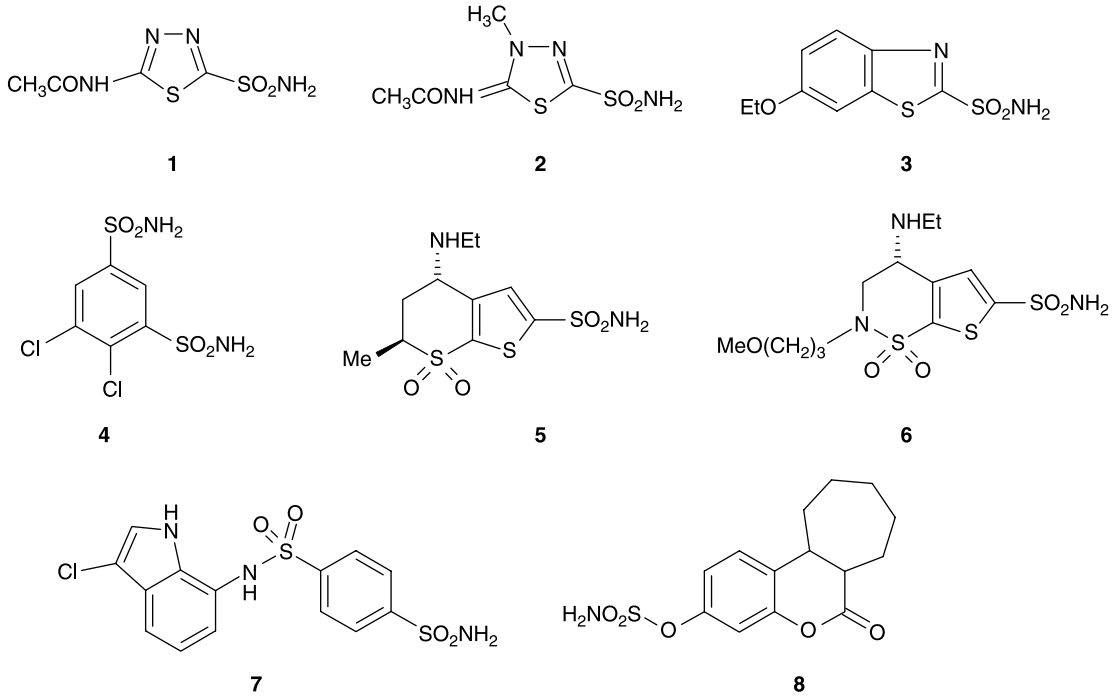
also show marked anticonvulsant, antiobesity and antitumor effects [1,2,5–11]. This is due to the fact that such inhibitors target different isozymes among the 16 presently known in vertebrates [1,2]. However, most of the presently available CAIs show undesired side effects due to indiscriminate inhibition of CA isoforms other than the target one.[1,2,5–14] Thus, many new CAI classes are being developed in the search of isozyme-selective compounds as potential drugs with less side effects [1,5,25].

them, some sulfamates and sulfonamides were characterized by X-ray crystallography and homology modelling [1,2,8–11]. Heterocyclic, aromatic sulfonamides as well as aliphatic sulfonamides/sulfamates/-sulfamides possessing low nanomolar inhibitory activity against CA IX have been detected so far [1,2,7,9–11].

As described above, hypoxia, through the HIF cascade, leads to a strong over-expression of CA IX in many tumors. The overall consequence of this is a pH



The isoform CA IX expression was shown to be strongly increased in many types of tumors, such as gliomas/ependymomas, mesotheliomas, papillary/follicular carcinomas, as well as carcinomas of the bladder, uterine cervix, kidneys, esophagus, lungs, head and neck, breast, brain, vulva, and squamous/basal cell carcinomas, among others [1,2,7,9–11]. In some cancer cells, due to the fact that the von Hippel-Lindau (VHL) gene is mutated, a strong upregulation of CA IX (up to 150-fold) as a consequence of constitutive hypoxia inducible factor (HIF) activation has been reported [1,2,8–11].

CA IX belongs to the highly active human α-CAs, its catalytic properties for the CO_2 hydration reaction being comparable with those of the highly evolved catalyst CA II [1,2]. As for all α-CAs, CA IX is susceptible to inhibition by anions and sulfonamides/sulfamates, with the inhibitors coordinating directly to the zinc ion within the active site cavity and participating in various other favorable interactions with amino acid residues situated both in the hydrophobic and hydrophilic halves of the active site [1,2,7,9–11]. Many low nanomolar CA IX inhibitors have been identified in the last several years. Among

imbalance, with most hypoxic tumors having acidic pH values around 6, in contrast to normal tissue which has characteristic pH values around 7.4 [9–11]. Constitutive expression of human CA IX was recently shown to decrease extracellular pH (pH_e) in Madin-Darby canine kidney (MDCK) epithelial cells.[8–11] CA IX selective sulfonamide inhibitors were also shown to reduced the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H^+ ions, binding specifically only to hypoxic cells expressing CA IX. Deletion of the CA active site was also shown to reduce the medium acidity, but a sulfonamide inhibitor did not bind to the active site of such mutant proteins. Therefore, tumor cells decrease their pH_e both by production of lactic acid (due to the high glycolysis rates), and by CO_2 hydration catalyzed by the tumor-associated CA IX, possessing an extracellular catalytic domain. Low pH_e has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, induction of the expression of cell growth factors and protease activation. CA IX probably also plays a role in providing bicarbonate to be used as a substrate for cell growth, whilst it is established that

Table I. Elemental analysis data for the new compounds **11-14** reported here.

No	Mp (°C)	Yield (%)	Elem anal. (calc,/found)			
			% C	% H	%N	%S
11	>300	75	46.30/46.27	3.53/3.50	9.00/8.96	20.57/20.52
12	168	80	49.55/49.36	4.39/4.42	8.25/8.29	18.87/18.90
13	>300	85	33.92/33.88	2.59/2.40	9.89/9.50	22.61/22.51
14	>300	67	27.00/26.91	2.25/2.03	15.75/15.50	27.00/26.85

bicarbonate is required in the synthesis of pyrimidine nucleotides [8–11].

Considering the fact that CA IX (as well as the second tumor-associated isozyme CA XII) [1,2] were recently shown to be druggable targets, we report here the synthesis, CA inhibitory activity and cytotoxic effects against some tumor cell lines *in vitro*, of a small series of biphenyl-disulfonamide derivatives.

Materials and methods

Chemistry

Sulfonamides of type **10**, 4,4′-biphenyl-disulfonyl chloride **9**, solvents and inorganic reagents were of highest purity available from Sigma-Aldrich (Milan, Italy). Enzymes were recombinant forms obtained as reported earlier by our group [8–12].

Synthesis of derivatives 11-14:. A mixture of 0.1 mole of 4,4′-biphenyl-disulfonyl chloride [26] 0.2 mole of 4-aminobenzenesulfonamide, 4-amino-ethyl-benzenesulfonamide, 6-chloro-4-aminoben-zene-1,3-disulfonamide or 5-amino-1,3,4-thia-diazole-2-sulfonamide, and 0.2 moles of pyridine were refluxed in dry ether (250 mL) for 4 h [26,27]. In case of synthesis of 5-amino-1,3,4-thiadiazole-2-sulfonamide derivative, the Scotten-Baumann conditions were used, and the corresponding amine was dissolved in 15 mL solution 2.5 M NaOH and cooled to 2–5°C in a salt-ice bathe. After the reactions were completed, the solvent was evaporated in vacuo, adjusted to pH 2 with 5 N HCL, and the precipitated bis-sulfonamides were filtered and recrystallized from aqueous ethanol to give biphenyl-4,4′-disulphonamide derivatives **11-14**. The chemical structures were confirmed by elemental analysis (Table I), FTIR spectroscopy (Table II), NMR spectroscopy and mass spectra. ^{1}H-NMR of **11**: δ 11.0 (s, 2H), 7.9 (s, 8H), 7.7 (m, 8H), 7.3 (d, 4H, $\mathcal{J} = 8.8$ Hz); MS: molecular ion peak (m/e): 622 g/mol; ^{1}H-NMR of **12**: δ 7.9 (m, 4H), 7.9 (m, 6H), 7.7(d, 4H, $\mathcal{J} = 8.2$ Hz), 7.4 (d, 4H, $\mathcal{J} = 8.2$ Hz), 7.3 (s, 4H), 3.1 (m, 4H), 2.8 (m, 4H); MS: molecular ion peak (m/e): 678 g/mol; ^{1}H-NMR of **13**: δ 8.2 (s, 2H), 7.6 (s, 6H), 7.4 (s, 6H), 7.0 (s, 2H), 6.6 (s, 6H); MS: molecular ion peak (m/e): 849 g/mol; ^{1}H-NMR of **14**: δ 7.75–7.70 (m, 12H), 6.80 (s, 2H), MS: molecular ion peak (m/e): 711 g/mol.

CA assay:

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity [28]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier [12–14] and represent the mean from at least three different determinations. Enzyme concentrations in the assay system were in the range of 7.1- 13 nM.

Table II. FTIR spectroscopy of 4,4′ biphenyl disulphonamide derivatives **11-14**.

No	Ar (CH)	SO_2	NH	NH_2	CH2
		cm^{-1}			
11	3053	1140, 1330	3250	3359, 3390	–
12	3060	1150, 1330	3276	3290, 3385	2790
13	3082	1142, 1315	3248	3277, 3374	–
14	3058	1187,1342	3255	3270,3394	–

Antiproliferative assay:

Stock solutions of inhibitor (1 mM) were prepared in DMSO, and dilutions up to 10 nM done with distilled deionized water. The percentual growth (PG) of the cells in the presence of five –six concentrations (10^{-8}–10^{-4} M) of inhibitor was calculated according to one of the following two expressions (*1*) or (*2*):

$$PG = 100 \times (\text{Mean } OD_{test} - \text{Mean } OD_0)/(\text{Mean } OD_{ctrl} - \text{Mean } OD_0), \quad \text{when } (\text{Mean } OD_{test} - \text{Mean } OD_0) \geq 0, \quad (1)$$

$$PG = 100 \times (\text{Mean } OD_{test} - \text{Mean } OD_0)/\text{Mean } OD_0, \quad \text{when } (\text{Mean } OD_{test} - \text{Mean } OD_0) < 0, \quad (2)$$

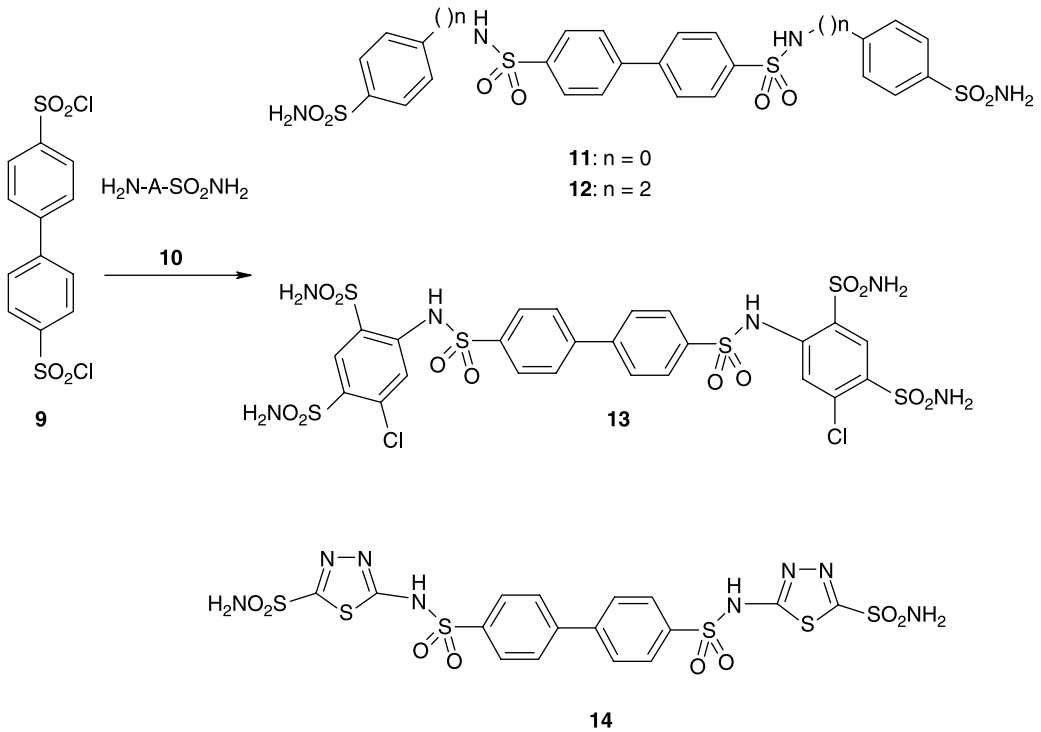
where: Mean OD_0 = the average optical density measurements of sulforhodamine B (SRB)-derived color just before exposure of cells to the test compounds; Mean OD_{test} = the average optical density measurements of SRB-derived color after 48 h exposure of cells to the test compounds; Mean OD_{ctrl} = the average optical density measurements of SRB-derived color after 48 h with no exposure of cells to the test compounds. GI_{50} represents the molarity of inhibitor producing a 50% inhibition of growth of the tumor cells after 48 h exposure to variable concentrations (10^{-4}–10^{-8} M) of the test compound, measured as outlined before, and this parameter was obtained by interpolation. GI_{50} represents the molarity of inhibitor at which PG = 50% [29]. The standard suforhodamine B (SRB) protein assay has been used to estimate cell viability or growth [29–31].

Results and discussion

Reaction of 4,4-biphenyl-disulfonyl chloride **9** [26] with aromatic/heterocyclic sulfonamides also incorporating a free amino group (in a molar ratio of 1:2), such as 4-aminobenzenesulfonamide, 4-aminoethyl-benzenesulfonamide, 6-chloro-4-aminobenzene-1,3-disulfonamide or 5-amino-1,3,4-thiadiazole-2-sulfonamide of type **10**, in the presence of base (pyridine or Schotten-Baumann conditions) [27] afforded the bis-sulfonamides **11-14** by the literature procedure [27] (Scheme 1). The new compounds have been characterized by standard procedures such as elemental analysis, IR, and NMR spectroscopy and MS, which conformed their structure (Tables I and II).

Derivatives **11-14** reported here and standard sulfonamide/sulfamate CAIs (of types **1-8**) were tested for the inhibition of four physiologically relevant mammalian CA isozymes, the human CA I and II (hCA I and hCA II), cytosolic, ubiquitous isoforms, as well as the transmembrane, tumor-associated isozymes hCA IX and XII (Table III).

The following SAR data may be drawn from Table III: (i) the slow cytosolic isozyme CA I was weakly inhibited by the biphenylsulfonamides **11-14**,



Scheme 1. Preparation of biphenylsufonamides **11-14**.

Table III. CA inhibition data of sulfonamides **11-14** reported in the present paper and standard inhibitors **1 - 8**, against isozymes I, II (cytosolic), IX and XII (transmembrane), by a stopped-flow, CO_2 hydration assay [28].

Inhibitor	K_I^* hCA I[a]	hCA II[a]	hCA IX[b]	hCA XII[b]
	(nM)	(nM)	(nM)	(nM)
1	250	12	25	5.7
2	50	14	27	3.4
3	25	8	34	22
4	1200	38	50	50
5	50000	9	52	3.5
6	45000	3	37	3.0
7	31	15	24	3.4
8	3450	21	34	12
11	675	64	59	145
12	604	43	23	213
13	1237	129	79	362
14	693	21	35	154

*Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay [28]; [a]Human, recombinant isozymes,; [b]Catalytic domain of human, cloned isoform [12,13].

with inhibition constants in the range of 604–1237 nM, similarly with some of the clinically ued derivatives, such as dichlorophenamide **4** or COUMATE **8**, which show K_Is in the same range as derivatives **11-14**. The best CA I inhibitor among the new derivatives was **12**, the 4-aminoethyl-benezenesulfonamide derivative, whereas the worst one was the 1,3-benzenedisulfonamide derivative **13**, which being probably much bulkier that **12** is around two fold lss active as a CA I inhibitor; (ii) against the rapid, housekeeping cytosolic isoform CA II, the new derivatives **11-14** showed moderate-good inhibitory capacity, with K_Is in the range of 21–129 nM (Table III). The best CA II inhbiitor was the 1,3,4-thiadiazole sulfonamide derivative **14** (with the same potency as the clinically used derivatives **1-8**) whereas the least active was again the bulkier 1,3-benzenedisulfonamide **13**, which was 6.1 times less effective an inhibitor as compared to **14**. The benzene-sulfonamides **11** and **12** had an intermdiate activity bwteen those of **13** and **14**, with the longer inhibitor **12** being slightly more effective as compared to the sulfanilamide compound **11**; (iii) a quite good inhibitory activity of compounds **11-14** was observed against the tumor-associated isoform CA IX, which has been inhibited with K_Is in the range of 23–79 nM. The best inhibitor in this case was again **12** (as for CA I), followed by **14** and **11**, whereas the worst one was the bulkier **13**. It should be observed that **12** and **14** have the same type of activity against this isoform as the clinically used derivatives indisulam **7** and COUMATE **8**, in phase II/III clinical development as antitumor drugs [1,2,7]. This flat SAR (i.e., a rather compact behavior of potent inhibitors for all the new derivatives **11-14**) is probably due to the fact that the CA IX active site is about 25% wider as compared to the CA II active site [12], based on homology modeling, as the X-ray crystal structure of the tumor-associated isozyme is not yet reported, explaining why relatively bulky inhibitors as the compounds investigated here may bind with facility to this siozyme. Indeed, for isoforms with a more restricted active site (such as CA I and II), the variation of K_I-s is much wider (i.e., between 21 and 129 nM against hCA II) as compared to the range we observed for the inhiibtion of hCA IX with the new compiuonds **11-14** reported here (between 23 and 79 nM, Table III); (iv) the second transmembrane, tumor-associated isoform, CA XII was much less inhibited by these compounds as compared to CA IX. In fact, the biphenylsulfonamides **11-14** showed inhibition constants in the range of 145-362 nM against this isoform, being much less effective inhibitors as compared to the clinically used sulfonamides/sulfamates **1-8** (K_Is in the low nanomolar range, see Table III); (v) data of Table III also show that three of the new compounds, i.e., **11-13**, are slightly more selective or the inhibition of CA IX over CA II, whereas **14** is a better CA II than CA IX inhibitor.

Compounds **11-14** reported here were screened for *in vitro* antiproliferative activity against the human colon cancer cell line HCT116, the human lung cancer cell line H460 and the human breast cancer cell line MCF-7 [29–31]. The concentration required for 50% cell growth inhibition (GI_{50}) was determined by the SRB (Sulpho Rodamine B dye) [29] coloremitric assay. As shown in table III these disulfonamide derivatives showed a good antiproliferative activity especially against the HCT116 line, with an GI_{50} in the range of 3.92–8.19 μg/mL. Theresults showed that the derivative **11** was the most active against the HCT116 cell line and H460 cell lines, with GI_{50} values of 3.29 μg/mL and 10 μg/mL, respectivily as shown in Figure 1A. The derivative **14** had a comparable antiproliferative activity against the human colon cell line, with an GI_{50} value of 3.789 μg/mL as shown in Figure 1D. The other two derivatives (**12** and **13**) showed a relatively less strong antiproliferative activity with GI_{50} value 0.74 μg/mL and 8.19 μg/mL respectively as shown in figures 1B and C.

In conclusion, we report here the synthesis of a small series of biphenyl-sulfonamides with CA inhibitory activity. The compounds were rather modest inhibitors of isozymes CA I and XII; but much more efficient as inhibitors of the cytosolic CA II and transmembrane, tumor.associated CA IX, with inhibition constants in the range of 21–129 nM gainst hCA II, and 23–79 nM against hCA IX, respectively. The compounds also showed inhibition of growth of several tumor cell lines (*ex vivo*), with GI_{50} values in the range of 0.74–10.0 μg/mL against the human colon cancer cell line HCT116, the human lung cancer cell line H460 and the human breast cancer cell line MCF-7.

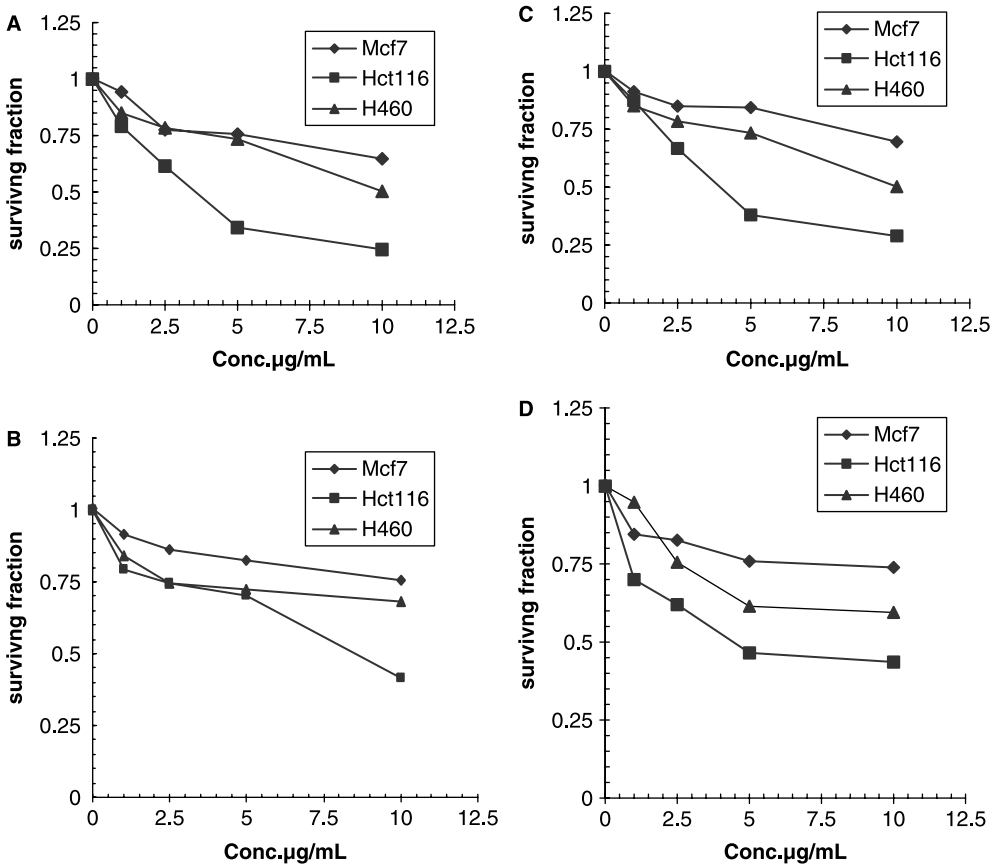


Figure 1. Inhibition of growth of three tumor cell lines (human colon cancer cell line HCT116, human lung cancer cell line H460 and human breast cancer cell line MCF-7) with compounds **11** (A), **12** (B), **13** (C) and **14** (D).

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