Cysteine-Modifying Agents: A Possible Approach for Effective Anticancer and Antiviral Drugs

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Modification of cysteine residues in proteins, due to *a*) the participation of the thiol moiety of this amino acid in oxido-reduction reactions, *b*) its ability to strongly coordinate transition metal ions, or *c*) its nucleophilic nature and facile reaction with electrophiles, may be critically important for the design of novel types of pharmacological agents. Application of such procedures recently led to the design of novel antivirals, mainly based on the reaction of zinc finger proteins with disulfides and related derivatives. This approach was particularly successful for developing novel antiviral agents for human immunodeficiency virus and human papilloma virus. Several new anticancer therapeutic approaches, mainly targeting tubulin, have also been reported. Thus, this unique amino acid offers very interesting possibilities for developing particularly useful pharmacological agents, which generally possess a completely different mechanism of action compared with classic agents in clinical use, thus avoiding major problems such as multidrug resistance (for antiviral and anticancer agents) or high toxicity. *Key words:* anticancer agent, antiviral agent, cysteine, thiol. *Environ Health Perspect* 110(suppl 5):801–806 (2002).
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Thiol groups tend to interact with metal ions, particularly with heavy metal ions. Consequently, cysteine (Cys) residues of proteins are often involved in binding, transport, and storage of metals in the cell, as well as in detoxification therapies (*1,2*). Among the 20 natural amino acids that constitute the building blocks of proteins, Cys is unique for at least three reasons, connected with the presence of a thiol moiety in its molecule, which confers special physicochemical properties to its derivatives. First, two such moieties incorporated in two Cys residues, present in the same protein or on different polypeptide chains, easily participate in oxido-reduction processes leading to the formation of disulfide (S—S) bonds. These are critical for the proper folding of many proteins (Scheme 1, Reaction 1) (*1,2*). *In vivo*, this process requires a sufficiently oxidizing environment and at least two catalytic proteins, endoplasmic reticulum oxidoreductin 1 protein (Ero1p) and protein disulfide isomerase, whose roles in protein folding were only recently investigated in some detail (*1*). Furthermore, the thiol moiety of Cys residues present in proteins may participate in other oxido-reduction processes, involving other oxidants present in the environment, such as oxygen and oxygen radicals (superoxide, peroxide, hydroperoxide, etc.), thiol reagents, glutathione (when a thiol/disulfide exchange reaction of the type shown in Scheme 1, Reaction 2), occurs or nitric oxide or some of its precursors such as nitrous acid (when *S*-nitrosothiols are formed; Scheme 1, Reaction 3), to mention only the most common ones (*1,2*). In all these processes the –SH moiety of the targeted Cys residue(s) is modified, leading to drastically changed properties of the corresponding protein, which may have important physiological/pharmacological consequences.

Second, the thiol moiety (in its deprotonated, thiolate form—the pK_a of –SH in Cys is around 8.5 but may be lowered up to 6 when this moiety is in the neighborhood of other amino acid residues) easily participates in coordination of transition metal ions (M^{x+}) important in physiological processes, such as Zn(II), Cu(II), Fe(III), and so forth (Scheme 1, Reaction 4) (*2–4*). A large number of metallo-enzymes incorporate Zn(II) or Cu(II) ions coordinated by one or several –SH groups belonging to Cys residues [e.g., DNA and RNA polymerases, aspartate transcarbamoylase, ornithine decarboxylase, alcohol dehydrogenase, carbonic anhydrase, various proteases, endoglucanase, β-lactamase II, etc. (*3,4*)], whereas the metallothioneins—proteins that contain around 60 amino acid residues, 20 of which are Cys, bind up to seven Zn(II) ions (and also copper, cadmium, iron, or other trace elements), playing important biological functions correlated with the transport and availability of zinc (and other metal) ions in organisms such as higher vertebrates (*2–4*). The metallothioneins are also involved in the acute intoxication with metal ions such as cadmium, copper, lead, and nickel (*4*). Furthermore, the zinc finger proteins in which a Zn(II) ion is coordinated by Cys and His residues constitute a large superfamily of nucleic acid binding proteins that are also one of the major subsets of transcription factors in viruses, prokaryotes, and eukaryotes (*4*).

$$R_1SH + R_2SH \xrightarrow{\text{Oxidizing agent}} R_1S - SR_2 + 2H^+ + 2e^- \quad [1]$$

$$R_1S^- + R_2SSR_3 \rightleftharpoons R_2S^- + R_1SSR_3 \quad [2]$$

$$RSH + HNO_2 \rightleftharpoons RSNO + H_2O \quad [3]$$

$$RSH + M^{x+} + nL \rightleftharpoons RS^-M^{x+}L_n + H^+ \quad [4]$$
(n = 1–3, L = Cys, His, Asp, Glu, etc.)

$$RSH + E^+ \rightleftharpoons RS - E + H^+ \quad [5]$$
(Alkylation, arylation, acylation, etc.)

Scheme 1.

Third, the –SH group of Cys, similarly to many other thiol moieties, has a strongly nucleophilic character (mainly in the thiolate form), which allows its easy derivatization by many electrophiles (E^+), such as haloacetates, maleimides, activated sulfonyl groups, etc. In all these cases, a modified Cys residue is obtained, which possesses completely different physicochemical properties (Scheme 1, Reaction 5) (*5*). The *S*-alkylation/arylation, *S*-acylation, or *S*-sulfonylation of Cys residues, caused by the last property mentioned above, is indeed of exceptional importance mainly for the design of novel anticancer therapies (*2,5*).

In this article, we review the many therapeutic applications that recently have been developed, considering the three above-mentioned properties of the Cys residues (i.e., their participation in oxido-reduction processes, their metal ion–coordinating properties, and their nucleophilicity and propensity to be alkylated/acylated or modified by means of other reagents). Such applications mainly deal with the design of novel antivirals and some new anticancer therapeutic approaches.

Antivirals

Anti-HIV Agents

Human immunodeficiency virus (HIV) infection affects more than 36 million people worldwide (*2,6*). Although much progress has been made in the treatment of HIV infection by the introduction of highly active antiretroviral therapy (HAART; a combination of nucleoside and nonnucleoside reverse transcriptase inhibitors, and/or

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aspartic protease inhibitors), the massive viral replication (with $>10^9$ virions produced daily) and the high error rate of the reverse transcriptase lead to the emergence of drug-resistant strains and the urgent need for new therapeutic approaches (*6*).

A new target for the development of anti-HIV therapies has been reported by Rice et al. to be the HIV-1 nucleocapsid (NC) zinc finger protein NCp7 (*7*). All retroviruses (except spumaretroviruses) contain in their NC proteins one or two copies of a conserved sequence motif termed the Cys array or the Cys–His box, composed the sequence C–$(X)_2$–C–$[X]_4$–H–$[X]_4$–C (C = cysteine, H = histidine, X = any amino acid; the number after the brackets is the number of amino acid residues between the two elements mentioned above) (*7–10*). This motif is also known as the NC zinc finger because a Zn(II) ion is tetrahedrally coordinated by the thiolate of the three Cys residues and the imidazole nitrogen belonging to the histidine residue (*7–10*). The NC proteins of all retroviruses are indeed essential for the viral life cycle because they select viral RNA from cellular RNA for dimerization and packaging, promote binding of the essential transfer RNA primer to the primer site, stimulate reverse transcription, and protect the viral RNA from nucleases, and their mutation/modification is not tolerated (*7–10*). Rice et al. (*7–10*) showed that it is possible to target NCp7 by chemically modifying the nucleophilic sulfur atoms coordinating the Zn(II) ions in the above-mentioned zinc fingers, with 3-nitrosobenzamide derivatives **1**, disulfide benzamides **2**, dithianes **3**, or azodicarbonamide **4**, among others, the process being followed by zinc extrusion from the zinc finger and production of noninfective virions. Furthermore, no resistance to such agents has been observed (*7–10*) because, as stated above, no mutations in the NC proteins are tolerated by retroviruses, HIV included.

The mechanism of action of such zinc finger–targeting agents of types **1–4** has been investigated in detail (*7–10*): the zinc is ejected in a stoichiometric, oxidative manner. Initially, the antiviral agent (i.e., a dithiobisamide **2**) forms an intermolecular disulfide bond (by means of a disulfide exchange reaction; Scheme 1, Reaction 2) because of an electrophilic attack on one of the sulfur atoms coordinating the Zn(II) of the zinc finger. This process leads to the destabilization of the Zn(II) ion coordination sphere and is followed by ejection of the metal ion from the zinc finger. The initial process may then be followed by the formation of intramolecular disulfides within the protein as well as disulfide bond rearrangements, but regardless, the final result is the denaturation of the zinc finger protein, which is lethal for the virus, leading to the formation of noninfective virions (*7–10*). It should be stressed that these compounds do not act as competitors per se for binding of zinc ions other than those belonging to zinc finger proteins. Some of the most active of such derivatives are shown in Table 1, together with second-generation compounds, belonging to the pyridinioalkanoyl thiolester type of derivative **5** in Table 1. Among the most effective antiviral derivatives are some sulfonamides (**2a**, **2c**, and **5a** in Table 1).

It has also been proven that some of these agents (**2** and **5** in Table 1) possess selectivity for the viral zinc finger protein (NCp7), without appreciably reacting with several cellular zinc fingers, such as the poly(ADP-ribose) polymerase and the Sp1 and GATA-1 transcription factors, nor did they inhibit HeLa nuclear extract–mediated transcription (*10*). This finding is of great importance in validating these compounds as valuable leads for the development of new antiviral drugs.

Antihuman Papilloma Virus Agents

Among the DNA viruses provoking debilitating disease responsible for significant mortality and morbidity, human papilloma

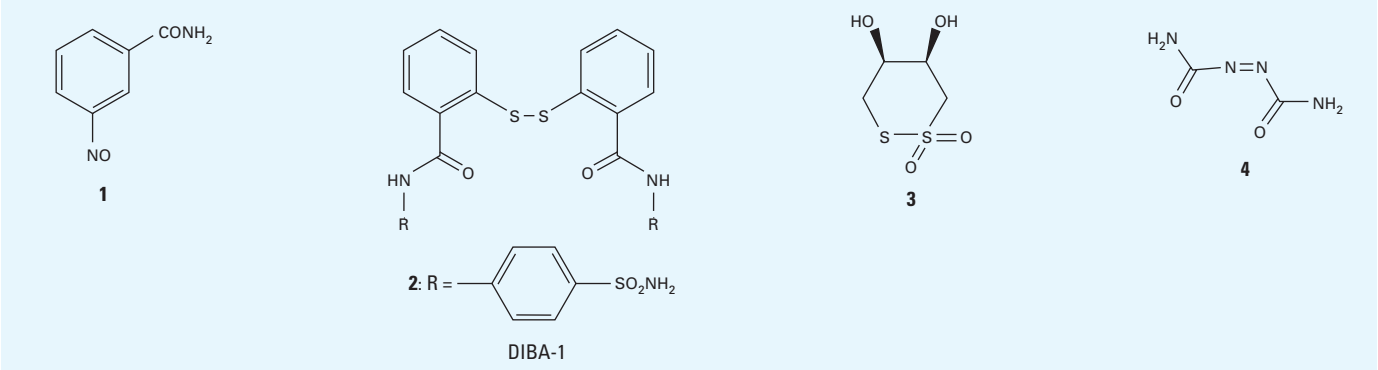


Table 1. Antiviral activity of compounds targeting NCp7.

Structure Type	RNH	EC_{50} (μM)[a]	IC_{50} (μM)[a]	Zinc finger reactivity	Structure
2a	4-NHC_6H_4–SO_2NH_2	0.85	217	Medium	
2b	4-NHC_6H_4–SO_2NHAc	1.5	> 200	Medium	
2c	4-$NHCH_2C_6H_4$–SO_2NH_2	1.4	30	Medium	
2d	4-NHC_6H_5	3.7	7	–	
2e	–NH-*i*-Bu	1.8	4.6	Medium	
2f	–NH-β-Ala	7.8	> 120	High	
2g	–NH-Val	5.0	140	High	
5a	4-$NHCH_2C_6H_4$–SO_2NH_2	1.1	55	1.4	
5b	4-NHC_6H_4–SO_2NHAc	6.2	> 316	3.6	
5c	4-NHC_6H_4–$SO_2C_6H_4$-4-NO_2	5.5	> 316	4.1	

[a]Antiviral activity was measured, by the XTT cytoprotection assay, as described elsewhere (*7–10*). EC_{50} represents the molarity of compound that provides 50% cytoprotection (prevention of HIV-induced cytopathicity), whereas IC_{50} represents the molarity of compound eliciting 50% cell death and is a measure of the toxicity of these derivatives.

viruses (HPVs) represent a special case. HPVs provoke different forms of benign and malignant mucocutaneous disease (common warts, plantar and palmar warts, genital warts, epidermodysplasia verruciformis) (*11*), and some of these viruses, such as HPV-16 and HPV-18, are also associated with cervical cancer, a particularly serious form of cancer (approximately a half million women worldwide die of this disease every year) (*11*). Currently, therapeutic options for HPV infections are limited, expensive, and ineffective (*11*), and they include surgery or immune modulators, such as interferon (*11*) and imiquimod (*2*).

Three proteins associated with oncogenesis promoted by HPV-16 have been isolated: E5, E6, and E7 (*12*). E6 and probably also E7 play an important role in crucial biochemical/cellular processes, such as transmembrane signaling, regulation of cell cycle, transformation of established cell lines, and ultimately oncogenesis (*12*). The E6 oncoprotein of HPV-16 contains 158 amino acid residues and incorporates two Cys–$[X]_2$–Cys–$[X]_{29}$–Cys–$[X]_2$–Cys zinc fingers (*12*). The structure and function of this oncoprotein are strongly dependent on these zinc fingers, which assure integrity and stability because of chelation of the metal ion by the four –SH moieties of the Cys residues mentioned above (*12*). Taking into account the successful design of zinc finger oxidants targeted against HIV-1 previously described, it was logical to extend this approach to other interesting viral proteins, such as E6. Beerheide et al. (*13,14*) have designed novel types of zinc finger chelators targeted against the E6 oncoprotein of HPV-16.

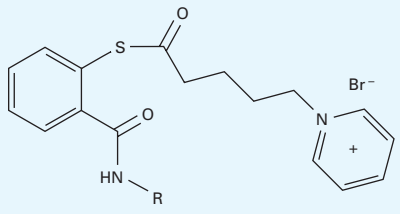
A large series of polysulfides such as **6** and **7** were prepared and tested *in vitro* for their ability to disrupt the integrity of chelated zinc ions present in the E6 HPV viral proteins. A very large number of substitution patterns for derivatives **6** and **7** have been investigated, with R^1, R^2 moieties being simple alkyls, cycloalkyls, aralkyls, hetaryls, and so on, or R^1R^2N in a cyclic moiety (e.g., azacyclopropyl, morpholyl, phthalimido, piperazinyl, etc.), as well as substituting such moieties with halogens, acyl, and aryl/alkyloxycarbonyl groups among others. The disulfides (n = 2 in **6**) were the most investigated and most active such derivatives. The viability of HPV-infected cells in the presence of the test compounds **6** and **7** has also been determined (*ex vivo*) (incubation with tetrazolium salt and measuring of the optical absorption, with or without test compound added). The most active anti-HPV compounds of this type produced a release of zinc from E6 of at least 30%, reduced the binding of the E6 protein to its coactivators E6AP or E6BP, and exhibited selective cytotoxicity toward the HPV-infected cells (*14*).

Thus, the disruption of a chelated metal ion domain in a protein (in this particular case a Zn(II) ion from a zinc finger protein) may represent a fundamentally new approach for the drug design of antiviral agents, not only for obtaining new antiretroviral drugs, but also for compounds targeted against DNA viruses.

Anticancer Agents Targeting Cys Residues of β-Tubulin and Carbonic Anhydrases

Several widely used antitumor drugs (e.g., the vinca alkaloids vincristine and vinblastine, colchicine, and paclitaxel) exert their action by binding to tubulin, a heterodimeric (αβ) protein that is the key component of microtubules and thus of the cytoskeleton, sustaining the cell shape and facilitating the movement and deposition of protein complexes, organelles, and membrane vesicles (*2,15*). Tubulins also play a crucial function in cell division, being among the major constituents of mitotic spindles, which are essential for chromosomal separation during mitosis (*15*). Several β-tubulin isoforms are known in humans (*15–17*). Some of the antitumor drugs mentioned above, such as vincristine, vinblastine, and colchicine, bind reversibly to β-tubulin in disassembled tubulin heterodimers, thereby interfering with polymerization (*15–17*). Other agents alkylate multiple Cys residues of β-tubulin and affect microtubule formation, but generally such compounds lacked any selectivity and could not be used as drugs. Only recently, two new classes of derivatives that specifically bind to a Cys residue of β-tubulin have been described and assayed for their antitumor properties (*15–17*).

Medina and colleagues (*15–17*) reported a series of perfluorophenylsulfonamide derivatives **8–12**, which strongly inhibited the growth of a variety of tumors, including



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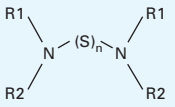
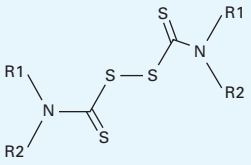


6: n = 2–6



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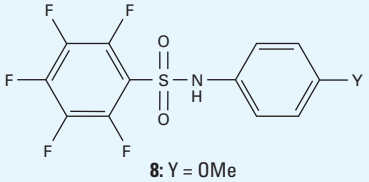
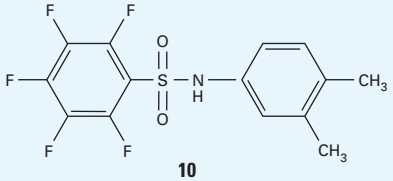


8: Y = OMe
9: Y = NMe_2



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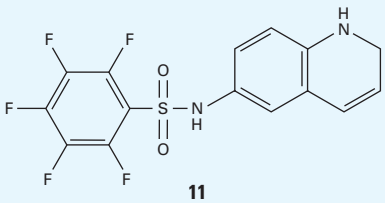

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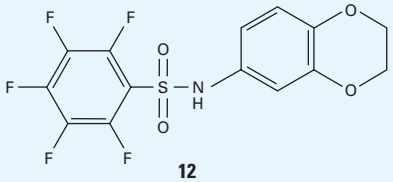

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multidrug-resistant cell lines. Compounds of this type possessed GI_{50} values (growth-inhibitory factor: the molarity of inhibitor producing a 50% reduction of growth of the tumor cells *in vitro*) in the low nanomolar range for derivatives **8** and **11**, and in the range of 40–500 nM for the other derivatives (*15*). Furthermore, the corresponding derivatives possessing other halogens instead of the five fluorine atoms of **8** have also been prepared, together with the corresponding isomeric trifluoro- and tetrafluorosulfonamides, but all these derivatives were much less active than the lead compound **8** (*17*). This was subsequently shown to be due to the mechanism of action of these compounds, which covalently bind to Cys-239 of β-tubulin, by means of a nucleophilic aromatic substitution reaction (*17*), in which the most substitutionally active fluorine atom of the antimitotic agent (i.e., the fluorine in *para* with the sulfonamide moiety) is displaced by the sulfur of Cys-239 residue of β-tubulin, leading to modification of the protein (Figure 1).

Scozzafava et al. (*18,19*) then reported a series of alkyl/arylsulfonyl-*N,N*-dialkyldithiocarbamates **13**, obtained by reaction of sodium *N,N*-dimethyldithiocarbamate or *N,N*-diethyldithiocarbamate with alkyl/arylsulfonyl halides. The reactivity of these derivatives against Cys and glutathione has been investigated to identify derivatives that might label a Cys residue of β-tubulin, similarly to compounds **8–12**. Some of the most reactive compounds against these thiol reagents also showed strong GI_{50} values (in the low micromolar range for some derivatives) against several cancer cell lines: leukemia, non–small-cell lung, ovarian, melanoma, colon, central nervous system, renal, prostate, and breast (Table 2). It has also been proved (*18,19*) that such compounds inhibit tubulin polymerization *in vitro*, probably by modification of the same Cys residue as in derivatives **8–12**, investigated by Medina and colleagues (*15–17*) (Figure 1).

At this point it is also worth mentioning the very strong antitumor properties of some sulfonamides of **14** and **15** (GI_{50} values in the low nanomolar range) reported by our group (*20–22*), which were initially designed as carbonic anhydrase inhibitors. Although the cellular target of these compounds is presently unknown, it is probable that they interfere with Cys residues of proteins involved in oncogenesis, such as carbonic anhydrase IX (*20–22*) or β-tubulin, because of the presence of the reactive thiocarbamoylsulfenylamino moieties in their molecules.

Conclusions

Cysteine, because of its unique properties among the 20 natural amino acids— connected with the presence of the thiol moiety in its molecule—offers the possibility of designing a host of therapeutic agents with interesting pharmacological applications. Such compounds interfere with one of the specific properties of this amino acid present in the target protein: *a*) the facile participation of Cys in oxido-reduction processes (leading to the formation of disulfide bonds); *b*) its high affinity for coordinating transition metal ions; or *c*) its high nucleophilicity and propensity to be modified (*S*-alkylated, *S*-acylated, *S*-sulfonylated, etc.).

Important applications have been registered in the design of antiviral agents, mainly targeted against HIV and HPV. Agents that interact with the HIV-1 NC zinc finger protein NCp7 were designed in such a way as to produce zinc ejection from the zinc finger, after oxidation of a Cys residue participating in coordination of the metal ion within the zinc finger. Because the NC proteins of all retroviruses, HIV included, are transcription factors that select viral RNA from cellular RNA for dimerization and packaging, and also promote binding of the essential transfer RNA primer to the primer site, stimulate reverse transcription, and protect the viral RNA from nucleases, these proteins are essential for the viral life cycle, and their mutation/modification is not tolerated. Thus, this approach may evade the drug resistance problems associated with HAART, the classical anti-HIV therapy, which targets the viral enzymes reverse transcriptase and aspartic protease, both subject to high rates of mutations that lead to emergence of drug resistance. The same approach of targeting viral zinc finger proteins has been applied for the design of anti-HPV agents, directed against the E6 oncoprotein of HPV-16, which incorporates two Cys–$[X]_2$–Cys–$[X]_{29}$–Cys–$[X]_2$–Cys zinc fingers. The designed antivirals produced zinc

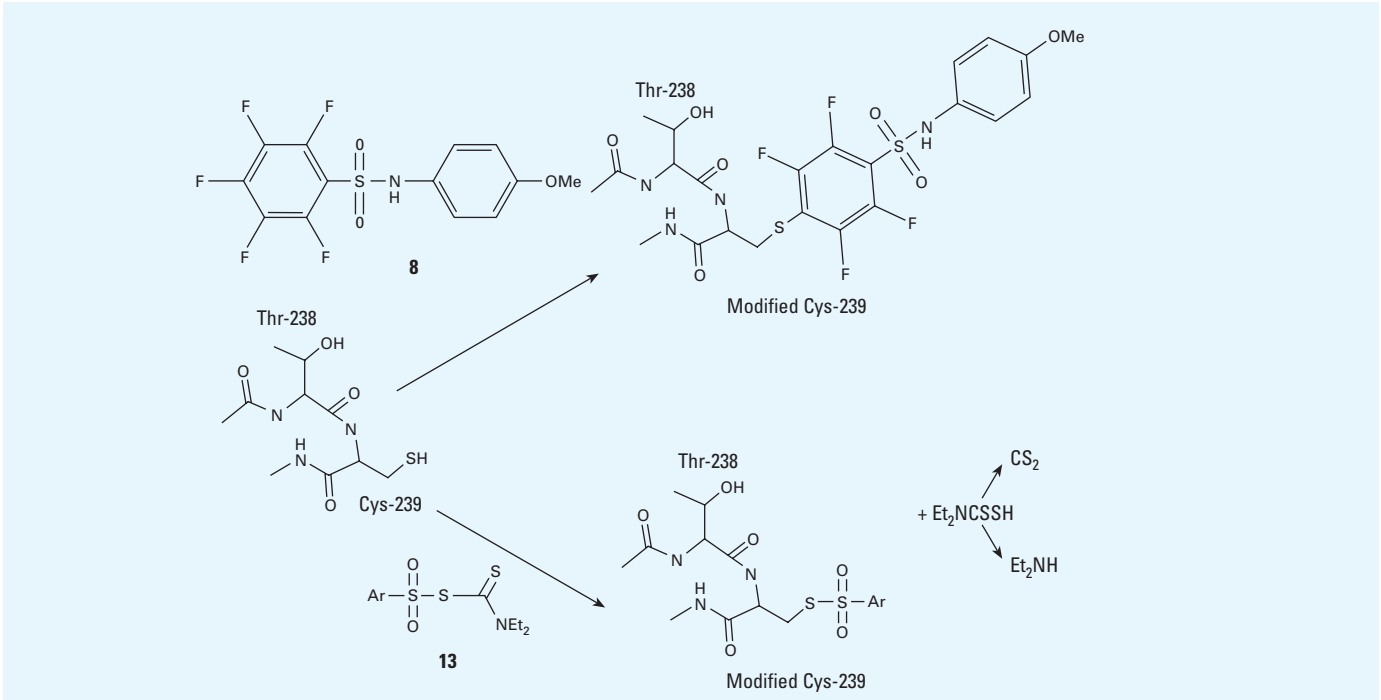

Figure 1. Modification of Cys-239 of tubulin β1, β2, or β4 by perfluorosulfonamide **8** (*15–17*) and alkyl/arylsulfonyl-*N,N*-diethyldithiocarbamates **13** (*18,19*).

Table 2. *In vitro* tumor growth inhibition data with compounds of the type shown in **13** (*18,19*).

		$RSO_2SC(=)NEt_2$ **13a-13e**				
		GI_{50} (μM)[a]				
Tumor cell line		**13a** *o*–HOOC–C_6H_4–	**13b** *o*–O_2N–C_6H_4–	**13c** *p*–O_2N–C_6H_4–	**13d** *p*–H_2N–C_6H_4–	**13e** *p*–AcNH–C_6H_4–
Leukemia	HL-60 (TB)	1.4	0.2	—	>100	24
	MOLT-4	—[b]	0.4	—	2	48
	K-562	—	11	19	34	69
	SR	3.6	0.3	0.1	3	0.01
	CCRF-CEM	82	2.5	—	>100	20
	RPMI-8226	—	15	12	22	18
Non–small-cell lung cancer	A549/ATCC	6.5	3	1	7	0.2
	HOP-62	>100	20	32	33	18
	HOP-92	>100	14	4	5	16
	NCI-H226	>100	36	83	98	35
	NCI-H522	1.2	0.08	0.02	1	<0.01
Colon cancer	COLO-205	>100	28	19	>100	22
	HCT-15	>100	34	52	>100	28
	HT29	>100	14	22	—	—
	SW-620	>100	15	18	>100	16
	HCC-2998	—	24	94	8	27
	HCT-116	—	15	5	>100	15
	KM12	—	12	11	22	13
Central nervous system cancer	SF-268	>100	26	>100	>100	44
	SF-295	>100	32	59	>100	28
	SF-539	—	20	21	—	—
	SNB-75	>100	76	77	48	32
	U251	>100	13	14	>100	15
Melanoma	LOX IMVI	>100	16	17	>100	14
	M14	>100	11	15	63	15
	MALME-3M	91	10	14	46	16
	UACC-257	>100	17	24	>100	9
	SK-MEL-28	>100	13	19	>100	15
	SK-MEL-5	37	3	2	13	0.4
Ovarian cancer	IGROV1	>100	0.5	—	5	12
	OVCAR-4	>100	23	45	>100	22
	OVCAR-3	>100	22	35	>100	31
	OVCAR-8	>100	18	20	>100	15
Renal cancer	768–0	>100	19	16	3	17
	ACHN	>100	24	36	61	27
	CAKI-1	>100	17	18	22	16
	RXF 393	>100	15	16	22	11
	UO-31	>100	13	21	80	20
	TK-10	—	29	43	>100	48
	SN12C	—	18	27	>100	18
Prostate cancer	PC-3	>100	54	—	90	—
	DU-145	>100	19	27	>100	23
Breast cancer	MCF7	>100	25	6	5	18
	MDA-MB-231/ATCC	>100	16	26	>100	20
	NCI/ADR-RES	—	30	48	>100	27
	MDA-N	>100	19	30	65	21
	HS 578T	—	29	>100	83	60
	MDA-MB-435	—	18	23	>100	19
	BT-549	—	26	36	>100	61

[a]Molarity of inhibitor producing a 50% inhibition of growth of the tumor cells after a 48-hr exposure to variable concentrations (10^{-4} to 10^{-8} M) of the test compound. [b]Compound has not been tested for the inhibition of growth of these tumor lines.

$RSO_2SC(=S)NEt_2$

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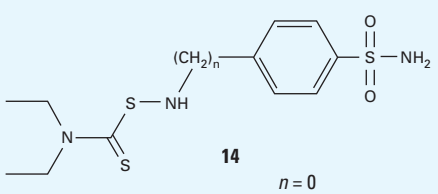

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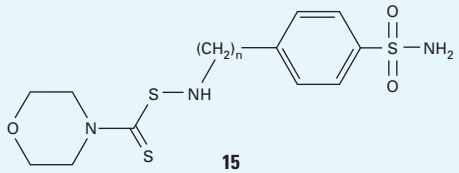
$n = 0$

$n = 1$

$n = 2$



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K_I = 10 – 35 nM (hCA II)

GI_{50} = 10 – 65 nM (different tumor cell lines)

ejection in the low micromolar concentration and strongly inhibited viral growth *in vitro*, leading to optimism regarding the possible development of successful antiviral therapies against this debilitating pathogenic agent.

Antitumor therapies that exploit the modification of Cys residues present in proteins critically involved in oncogenesis have also been reported recently. Several agents that target β-tubulin, such as pentafluorobenzenesulfonamide derivatives or alkyl/arylsulfonyl-*N,N*-dialkyldithiocarbamates, were shown to covalently bind to residue Cys-239 of β-tubulin, exerting in this way strong antitumor properties against a wide range of tumor cell lines (leukemia, non–small-cell lung, ovarian, melanoma, colon, central nervous system, renal, prostate, and breast cancer), including those with multidrug-resistant phenotype. Thus, a nonclassical antitumor agent that may evade the drug resistance problems correlated with the clinically used anticancer agents might be developed soon from this class of pharmacological agents.

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