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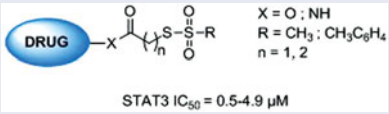
Methanethiosulfonate derivatives as ligands of the STAT3-SH2 domain

Elena Gabriele[a], Chiara Ricci[b], Fiorella Meneghetti[a], Nicola Ferri[c], Akira Asai[d] and Anna Sparatore[a]

[a]Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milano, Italy; [b]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milano, Italy; [c]Department of Pharmaceutical and Pharmacological Sciences, Università degli Studi di Padova, Padova, Italy; [d]Center for Drug Discovery, Graduate School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan

ABSTRACT

With the aim to discover new STAT3 direct inhibitors, potentially useful as anticancer agents, a set of methanethiosulfonate drug hybrids were synthesized. The *in vitro* tests showed that all the thiosulfonic compounds were able to strongly and selectively bind STAT3-SH2 domain, whereas the parent drugs were completely devoid of this ability. In addition, some of them showed a moderate antiproliferative activity on HCT-116 cancer cell line. These results suggest that methanethiosulfonate moiety can be considered a useful scaffold in the preparation of new direct STAT3 inhibitors. Interestingly, an unusual kind of organosulfur derivative, endowed with valuable antiproliferative activity, was occasionally isolated.



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Introduction

Signal transducer and activator of transcription 3 (STAT3) were identified in 1994 as a DNA-binding factor that selectively interacts with IL-6-responsive element in the promoter of acute-phase genes from IL-6-stimulated hepatocytes[1]. It belongs to STAT family, latent cytoplasmic transcription factors that are activated in response to extracellular signals such as cytokines, growth factors, and hormones. STATs directly transmit signals from plasma membrane to the nucleus and regulate cell growth and survival by modulating the expression of specific target genes. This family comprises seven isoforms, namely STAT1 to STAT4, STAT5a, STAT5b, and STAT6[2], that present several structurally and functionally conserved domains including the Src homology 2 (SH2) domain which is essential for the activation cascade pathway. Upon phosphorylation in the cytoplasm, STAT3 can dimerize forming homodimers or heterodimers through specific reciprocal SH2-phosphotyrosine interaction; the dimers then translocate into the nucleus, bind to specific DNA-binding elements, and activate transcription of target genes, which are mainly involved in cell proliferation, differentiation, apoptosis, and inflammation[3].

In addition, STAT3 was found to be constitutively activated by aberrant upstream tyrosine kinase activity in a broad spectrum of cancer cell lines and human tumors, and therefore it is considered a promising target for cancer therapy. Several studies confirmed that STAT3 inhibitors have minimal effects on normal cells[4,5], thus providing the potential for selective tumor cell elimination.

Two main approaches have been explored to inhibit STAT3 signaling[6,7]: direct, by interaction of small molecules with the protein and indirect, inhibiting the upstream tyrosine kinases that are responsible for STAT3 activation or blocking factors as JAK, Src, Bcr-Abl, FLT3, and EGFR that are involved in the activation of STAT3 signaling. This kind of inhibition induces tumor-cell apoptosis but it is poor selective.

Direct inhibitors should be preferred because the nonspecific mechanism of action of indirect inhibitors could cause important adverse effects. In the direct approach, much of the efforts have been addressed at disrupting the STAT3:STAT3 dimerization, which is a fundamental step in STAT3 activation. The slow progress of obtaining suitable direct STAT3 inhibitors for preclinical investigation and for clinical development could be attributed to the challenge of targeting protein-protein interactions (PPIs)[8], which are very different from those of more-established targets such as enzymes and G-protein-coupled receptors. Nonetheless, a number of successful examples started to prove that it is possible to overcome these hurdles and develop PPI modulators as drugs[9].

Recently, a number of small molecule compounds which directly inhibit the activity and function of STAT3 have been discovered and studied for cancer treatment and prevention[10,11].

Among them, **S3I-201** (Figure 1) has been identified as a selective STAT3-SH2 domain inhibitor, which blocked the formation of STAT3 homodimers ($IC_{50} = 86\ \mu M$) and inhibited the proliferation of breast and hepatocellular cancer cells in mice[12]. However, the presence of an electrophilic tosylate leaving group in **S3I-201** renders it highly susceptible to alkylate also other substrates, and therefore it is not very stable in the biological medium. Further studies lead to the synthesis of several analogs of **S3I-201**, able to bind the SH2 domain with higher affinity[13–15].

CONTACT Anna Sparatore anna.sparatore@unimi.it Department of Pharmaceutical Sciences, Università degli Studi di Milano, Via L. Mangiagalli, 5-20133 Milano, Italy

It is well known from literature that S-methyl methanethiosulfonate (SMMTS), isolated from cauliflower, is able to inhibit colon tumor incidence when administered to rats during the post-initiation phase of carcinogenesis[16].

Recently, we have synthesized and studied two methanethiosulfonate (MTS) derivatives (**1** and **2**; Figure 2)[17] of valproic acid (VA) endowed with interesting anticancer properties. In particular, compound **1** exhibited *in vitro* antiproliferative activity at micromolar concentration on different tumor cell lines[18,19] and *in vivo* inhibited the growth of PC3 in subcutaneous xenografts[18].

Although SMMTS[16] and other MTS derivatives[18,19] exert their chemopreventive and anticancer activity through multiple mechanism, their hypothetical direct or indirect activity on STAT3 had not been investigated yet. For this reason, to evaluate their ability to interact with STAT3-SH2 domain, we submitted compounds **1** and **2** to the AlphaScreen-based assay[20], an *in vitro* competitive binding test used to identify compounds able to directly inhibit the binding of SH2-containing proteins to their correspondent phosphopeptides, the physiological ligands.

Since both compounds showed a potent inhibition of the binding between STAT3-SH2 domain and its phosphopeptidic ligand, we decided to extend this investigation to other thiosulfonate-drug hybrids (Figure 2), as well as to their parent compounds, with the aim to better understand and confirm the behavior of the thiosulfonate moiety toward this protein.

NSAIDs-thiosulfonate hybrids **3**, **4**, and **5**, which are derivatives of sulindac, acetyl salicylic acid (ASA), and diclofenac, respectively, have been chosen because it is known that COX inhibitors are useful in the treatment of certain kind of tumors. The idea was that the combination of COX inhibition with the anticancer properties of thiosulfonates could lead to a new chemical entity where the two components act in a synergistic way against cancer development.

The anticancer activity of NSAIDs seems also related to additional mechanisms. Indeed, ASA induced apoptosis in colorectal cancer (CRC) cells in aspirin-treated mice[21] or in human glioblastoma cell line A172 via downregulation of IL-6-dependent STAT3 signaling[22] suggesting that aspirin could be useful for a potential anti-glioblastoma or anti-CRC therapeutic approach. Also sulindac treatment exerted a significant time-dependent cell growth-inhibitory effect on oral squamous cell carcinoma (SCCa) cells inducing a STAT3 down-modulation[23]. Since the above-mentioned activities seem to be related to the downregulation of STAT3 pathway and not to a direct interaction with the STAT3-SH2 domain, we thought that the linkage of a NSAID drug with a direct STAT3 inhibitor, such as a thiosulfonate derivative, could be a useful strategy to obtain a more powerful STAT3 inhibitor.

In addition, we decided to modify the structure of compound **S3I-201** through the replacement of the oxygen with a sulfur atom, thus obtaining compound **6** (Figure 1) or through the replacement of the tosylate group with the methanethiosulfonate (compound **7**, Figure 1). The aim was to evaluate if the presence of the thiosulfonate moiety can modify both the ability of **S3I-201** to interact with STAT3 and its potency as antiproliferative agent. Actually, compound **7** was not obtained, and compound **8** was instead isolated.

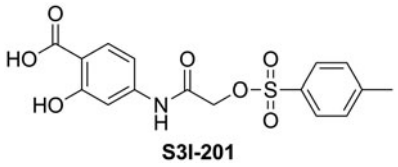


Figure 1. Structure of S3I-201.

Materials and methods

General

All commercially available solvents and reagents were used without further purification, unless otherwise stated. Reactions monitored by thin-layer chromatography (TLC) analysis on aluminum-backed Silica Gel 60 plates (70–230 mesh, Merck). CC = flash column chromatography (Geduran® Si 60, 40–63 μm, Merck). ^{1}H-NMR and ^{13}C NMR spectra: Bruker DRX Avance 300 MHz or Varian 300 MHz Oxford equipped with a non-reverse probe at 25 °C; $CDCl_3$, DMSO-d_6, D_2O; δ in ppm, *J* in Hertz. High-resolution mass spectra (HRMS): FT-Orbitrap mass spectrometer in positive/

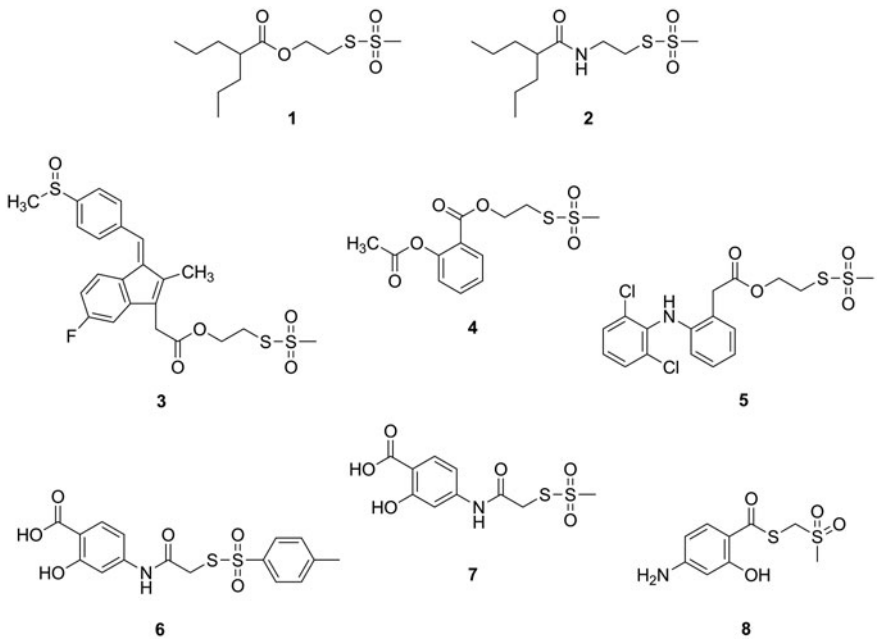


Figure 2. Structures of the studied thiosulfonate drug hybrids and of compound 8.

negative electro spray ionization (ESI). Melting points: Büchi Melting Point B540 instrument, uncorrected.

Synthesis of hybrid compounds (1), (2) and (5)

2-((Methylsulfonyl)thio)ethyl 2-propylpentanoate (**1**), S-(2-(2-propylpentanamido)ethyl) methanesulfonothioate (**2**) and 2-((methylsulfonyl)thio)ethyl 2-(2-((2,6-dichlorophenyl)amino)phenyl)acetate (**5**) were prepared according to the literature procedures[17,24].

(Z)-5-fluoro-2-methyl-1-[[4-(methylsulfinyl)phenyl]methylene]-1H-indene-3-acetic acid 2-methanesulfonylsulfanylethyl ester (3) and 2-acetoxybenzoic acid 2-methanesulfonylsulfanyl-ethyl ester (4)

General method

A 1 N solution of dicyclohexylcarbodiimide (DCC, 4.22 ml) in CH_2Cl_2 was added to a solution of S-(2-hydroxyethyl) methanesulfonothioate[25] (**9**; 3.84 mmol), 4-dimethylaminopyridine (DMAP, 0.18 mmol), and sulindac or acetyl salicylic acid (3.84 mmol) in CH_2Cl_2 (67 ml), and the mixture was stirred for 1.5 h at room temperature, under nitrogen. At the end of the reaction, the dicyclohexylurea (DCU) was filtered and the solution was extracted successively with a solution of 1 N HCl, afterward with water, then with a saturated solution of $NaHCO_3$ and water. Finally, the organic phase was dried on anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by CC on silica gel as indicated for each compound.

The two compounds have been already described in two patents[26,27], and their characterization is now integrated.

(Z)-5-fluoro-2-methyl-1-[[4-(methylsulfinyl)phenyl]methylene]-1H-indene-3-acetic acid 2-methanesulfonylsulfanylethyl ester (3)[26]

CC (CH_2Cl_2/MeOH, 99.5:0.5). Yield 80%; mp 118.5–119.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.70 (dd, 4H, CH_3SO_2-Ar-*H*); 7.20 (s, 1H, F-Ar-*H*); 7.18 (d, 1H, F-Ar-*H*); 6.85 (d, 1H, F-Ar-*H*); 6.58 (t, 1H, C = C*H*-); 4.40 (t, 2H, OCH_2); 3.60 (s, 2H, Ar-CH_2-COO); 3.40 (t, 2H, CH_2-S); 3.30 (s, 3H, SO_2-CH_3); 2.80 (s, 3H, SO-CH_3); 2.20 (s, 3H, -CH_3).

2-Acetoxybenzoic acid 2-methanesulfonylsulfanyl-ethyl ester (4)[27]

CC (cycloexhane/EtOAc, in gradient from 80:20 to 60:40). Yield 28%; mp 90.5–91.3 °C. ^{1}H NMR (300 MHz, $CDCl_3$: δ 8.00 (d, 1H, Ar-*H*); 7.60 (t, 1H, Ar-*H*); 7.30 (t, 1H, Ar-*H*); 7.10 (d, 1H, Ar-*H*); 4.60 (t, 2H, COO-CH_2); 3.50 (t, 2H, CH_2-S); 3.40 (s, 3H, SO_2-CH_3); 2.40 (s, 3H, OCO-CH_3).

2-((Methylsulfonyl)thio)acetic acid (15)

Sodium methanethiosulfonate (**13**; 500 mg, 3.73 mmol) and 2-bromoacetic acid (**14**; 470 mg, 3.38 mmol) were mixed together in acetone (8 ml). The reaction was stirred at room temperature for 20 h and was monitored by TLC. After the completion of reaction, inorganic salts were filtered and the solution was evaporated under reduced pressure. The resulting yellow pale oil was crystallized with CH_2Cl_2 to provide the final product as a white crystal solid. Yield 80%; mp 80.1–81.5 °C (Lit. 95–96)[28]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.18 (br s, 1H, -COOH collapsed with D_2O), 4.07 (s, 2H, -CO-CH_2-S), 3.53 (s, 3H, -CH_3) ppm.

2-Hydroxy-4-(2-(tosylthio)acetamido)benzoic acid (6)

Sodium toluenethiosulfonate[29] (**12**; 812 mg, 3.83 mmol) and 4-(2-chloroacetamido)-2-hydroxybenzoic acid[30] (**11**; 805 mg, 3.48 mmol) were dissolved in anhydrous DMF (7.5 ml) under nitrogen. The reaction was stirred at 60 °C for 4 h and was monitored by TLC. After cooling at room temperature, inorganic salts were filtered and the solution was evaporated under reduced pressure. The obtained residue was diluted with CH_2Cl_2 and washed with cold water three times and then with iced brine. The organic layer was dried with anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to dryness to provide a residue that was purified by CC (silica gel; CH_2Cl_2/MeOH; in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 1.3% of MeOH. A salmon pink solid was obtained. The solid was rinsed first with ethyl ether/petroleum ether (2:1) and then with ethyl ether/MeOH (1:0.2) to give the final product as a white solid. Yield 60%; mp 170.0–171.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.41 (br s, 1H, -OH collapsed with D_2O), 10.42 (s, 1H, -N*H*- collapsed with D_2O), 7.80 (d, 2H, J = 7.8 Hz, Ar-*H*), 7.71 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.38 (d, 2H, J = 7.8 Hz, Ar-*H*), 7.11 (d, 1H, J = 1.6 Hz, Ar-*H*), 6.91 (dd, 1H, J_1 = 1.6 Hz, J_2 = 8.8 Hz, Ar-*H*), 4.05 (s, 2H, -CH_2-S-), 2.31 (s, 3H, Ar-CH_3) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 171.93, 165.09, 162.41, 145.56, 145.01, 141.48, 131.47, 130.47, 127.31, 110.67, 108.53, 106.59, 21.44 ppm. HRMS (ESI): *m/z* calcd for $C_{16}H_{16}NO_6S_2$ $[M+H]^+$: 382.04190; found: 382.04130.

S-((methylsulfonyl)methyl) 4-amino-2-hydroxybenzothioate (8)

To a solution of 2-((methylsulfonyl)thio)acetic acid (**15**; 366 mg, 2.15 mmol) in anhydrous DMF (2 ml) under argon and at 0 °C, hydroxybenzotriazole (HOBt, 290 mg, 2.15 mmol), *N*,*N*-diisopropylethylamine (DIPEA, 0.341 ml, 1.96 mmol), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl, 412 mg, 2.15 mmol) were added. After 5-min *p*-aminosalicylic acid (300 mg, 1.96 mmol) was added, and the reaction was stirred at room temperature for 6 h. The reaction was monitored by TLC. After the completion of reaction, the solution was evaporated under reduced pressure. The obtained residue was diluted with EtOAc and washed first with a cold solution of 0.5 N HCl and then with cold brine. The organic layer was dried with anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to dryness, and the crude product was then purified by CC (silica gel; CH_2Cl_2/MeOH; in gradient); the product eluted with 0.3% of MeOH. After washing with diethyl ether, a white solid was obtained. Yield 6%; mp 199.6–202.3 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.67 (br s, 1H, OH collapsed with D_2O), 7.51 (d, 1H, J = 8.7 Hz, Ar-*H*), 6.43 (br s, 2H, NH_2 collapsed with D_2O), 6.17 (dd, 1H, J_1 = 2.1 Hz and J_2 = 8.7 Hz, Ar-*H*), 6.01 (d, 1H, J = 2.1 Hz, Ar-*H*), 4.66 (s, 2H, S-CH_2-SO_2), 2.98 (s, 3H, SO_2-CH_3) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 184.69, 161.68, 157.19, 131.72, 109.25, 107.76, 98.72, 49.48, 39.86 ppm. HRMS (ESI): *m/z* calcd for $C_9H_{11}NO_4S_2Na$ $[M+Na]^+$: 284.0027; found: 284.0024; calcd for $C_9H_{12}NO_4S_2$ $[M+H]^+$: 262.0208; found 262.02044; calcd for $C_9H_{10}NO_4S_2$ $[M\text{-}H^+]^-$: 260.0057; found 260.0051.

Single crystal X-ray analysis (8)

Crystals of compound **8** were obtained as colorless prisms from water/methanol (1:1) solution at room temperature, respectively. Intensity data were collected at room temperature on a Bruker Apex II CCD diffractometer, using graphite-monochromatized Mo-Kα radiation (λ = 0.71073 Å). Intensity data were corrected for Lorentz-polarization effects and for absorption (*SADABS*)[31]. The structures were solved by direct methods (*SIR97*)[32] and completed by iterative cycles of full-matrix least squares refinement on F_o^2 and Δ*F* synthesis using the *SHELX-97*[33] program (*WinGX* suite)[34].

The positions of hydrogen atoms were introduced by a close examination of a final difference Fourier.

These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Center, 12, Union Road, Cambridge CB21EZ, UK; fax: ++44 1223 336 033; or deposit@ccdc.cam.ac.uk). CCDC-1495448 number contains the supplementary crystallographic data for this paper.

Crystal data for (8)

$C_9H_{11}NO_4S_2$, $M_r = 261.3$ g/mol, Triclinic, Space group *P*-1, $a = 5.080(3)$ Å, $b = 9.8552(6)$ Å, $c = 11.4649(7)$ Å, $\alpha = 81.569(1)$, $\beta = 88.232(1)$, $\gamma = 77.41(1)$, $V = 554.12(6)$ Å^3, $Z = 2$, $D_{calc} = 1.566$ Mg/m^3, $R = 0.033$ (5853 reflections), $wR2 = 0.0864$, $T = 293(2)$K, $GOF = 1.080$. The reflections were collected in the range $1.8^\circ \leq \theta \leq 25.02^\circ$ employing a $0.55 \times 0.22 \times 0.02$ mm crystal.

AlphaScreen-based assay

STAT3 inhibitory activity of the described compounds was tested by the AlphaScreen-based assay to evaluate the potential inhibition of the interaction between STAT3-SH2 domain and pTyr-containing peptides according to the previously reported procedure[20]. For the most interesting compounds, selectivity tests versus STAT1 and Grb2 (Growth factor receptor-bound protein 2) were performed. AlphaScreen is a bead-based nonradioactive assay system for detecting biomolecular interactions in a microtiter plate format. Binding of biological partners brings donor and acceptor beads into close proximity and as result, a luminescent signal between 520 and 620 nm is produced. The AlphaScreen-based assays were typically performed in a final reaction volume of 25 µl of the assay buffer containing 10 mM HEPES–NaOH (pH 7.4), 50 mM NaCl, 1 mM EDTA (pH 8.0), 0.1% NP-40, and 10 ng/µl BSA in a 96-well microtiter plate at 25 °C. Phospho-Tyr (pTyr) peptide probes used in this study were 5-carboxyfluorescein (FITC)-GpYLPQTV for STAT3, FITC-GpYDKPHVL for STAT1, and FITC-PSpYVNVQN for Grb2. First, 75 nM of each SH2-containing protein was incubated with the test compound for 15 min. Each protein sample was then incubated for 90 min with 50 nM of its corresponding FITC-pTyr peptide, and mixed with streptavidin-coated donor beads and anti-FITC acceptor beads simultaneously before detection at 570 nm using EnVisonXcite (Perkin Elmer, Waltham, MA).

Cell culture

The colorectal cancer HCT116 cells were cultured in McCoy's media supplemented with penicillin (10,000 U/ml), streptomycin (10 mg/ml), nonessential amino acid, and 10% fetal calf serum (FCS). Cells were then seeded in 48 well plates and, after 24 h, they were incubated with different concentration of newly synthesized compounds dissolved in DMSO. The same volume of solvent was added to control conditions and did not exceed 0.5% (v/v).

MTT assay

After 48 h of treatment, medium was removed from each well, and cells were incubated with fresh medium, containing 10% (v/v) of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reagent. MTT, by interacting with vital cells mitochondria, is converted to formazan, with the development of visible purple crystals. After dissolving these crystals with isopropanol-HCl, the quantification of the conversion of MTT to formazan is determined through spectrophotometer analysis (595 nM), as previously described[35].

Results and discussion

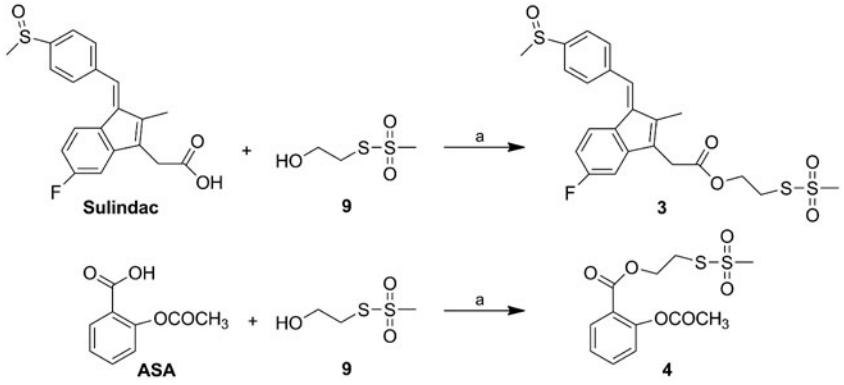
Synthesis

Compounds **1**, **2**, and **5** were prepared through coupling reactions between valproic acid and S-(2-hydroxyethyl) methanesulfonothioate (**9**) or S-(2-aminoethyl) methanesulfonothioate (**10**) or between diclofenac and **9**, respectively as previously described[17,24]. A similar synthetic route was used for the synthesis of **3**[26] and **4**[27] as indicated in Scheme 1.

Compound **6** was obtained by reacting sodium toluenethiosulfonate (**12**), prepared accordingly to[29], with 4-(2-chloroacetamido)-2-hydroxybenzoic acid (**11**; Scheme 2). This latter was prepared from the reaction of *p*-aminosalicylic acid with chloroacetyl chloride as described by Harte and Gunnlaugsson[30].

Since the analogous reaction of sodium methanethiosulfonate (**13**) with 4-(2-chloroacetamido)-2-hydroxybenzoic acid (**11**) did not afford the desired 2-hydroxy-4-(2-((methylsulfonyl)thio)acetamido)benzoic acid (**7**), we tried to obtain it through a coupling reaction between *p*-aminosalicylic acid (**PAS**) and 2-((methylsulfonyl)thio)acetic acid (**15**). This latter was prepared by reacting compound **13** with bromoacetic acid (**14**), following with few modifications the methods previously described[36,37] (Scheme 3). Surprisingly, instead of the methanethiosulfonate derivative (**7**), S-((methylsulfonyl)methyl) 4-amino-2-hydroxybenzothioate (**8**) was obtained in low yield through an, at the present, undefined mechanism possibly involving a concerted decarboxylation and rearrangement of the methanesulfonylthioacetic acid (**15**).

In order to unambiguously assess the molecular structure, we performed on compound **8** the crystallographic analysis and the



Scheme 1. Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, rt, 1.5 h.

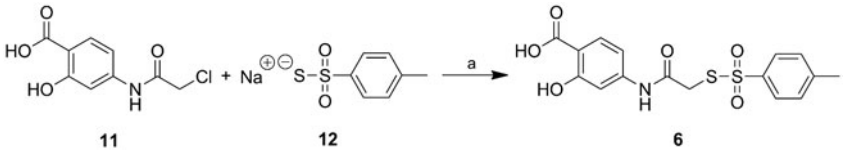
ORTEP[34] drawing is shown in Figure 3. In the molecule, the central -S–C = O moiety is approximately coplanar with respect to the *p*-aminosalicylic system, which presents a strong intramolecular O-H … O hydrogen bond. The conformation of the lateral chain is characterized by a torsion angle C7-S2-C8-S1 of 36(1)°. In the crystal, molecules are linked by N-H … O hydrogen-bonding interactions, forming chains parallel to the *b*-axis direction.

Biological results

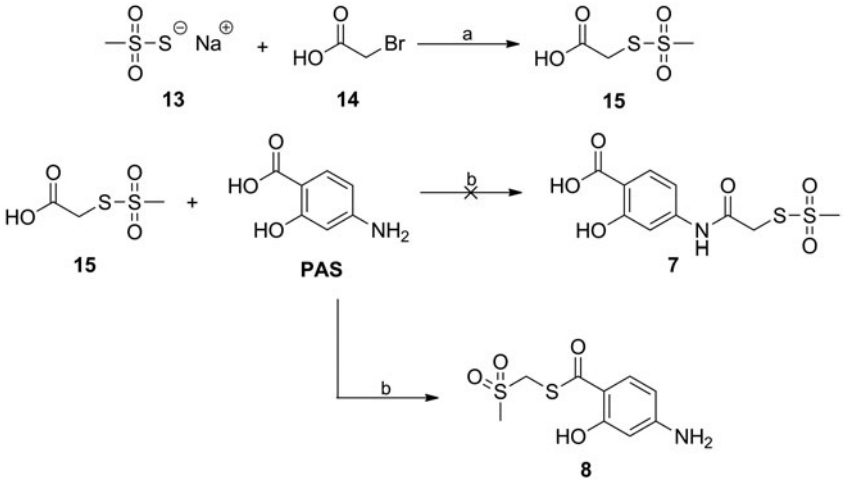
The thiosulfonate-drug hybrids **1–5**, the **S3I-201** analog **6** and compound **8**, together with their parent compounds have been submitted to the AlphaScreen-based assay, to investigate their ability to directly bind STAT3-SH2 domain.

Moreover, in order to check the selectivity of our molecules on STAT3, other SH2-containing proteins, such as STAT1 and Gbr2 (Growth factor receptor-bound protein 2), highly homolog to STAT3 (78% and 65%, respectively), have also been tested. Results, expressed as % of protein inhibition at 30 and 3 μM concentration or as IC_{50} (μM), are reported in Table 1. In addition, the cytotoxicity[35] of these compounds on HCT-116 cell line (a human colon carcinoma cell line which expresses high levels of STAT3[38]) was also tested. The inhibitory activities (IC_{50} μM values) are also listed in Table 1.

The obtained results indicate that all thiosulfonate hybrids are able to strongly and selectively bind STAT3-SH2 domain, whereas the parent drugs were completely devoid of this activity at the highest concentration tested (30 μM). Although NSAIDs such as acetylsalicylic acid and sulindac are reported to induce STAT3 downregulation[22,23], these results indicate that these drugs are not able to directly bind to the SH2 domain of the protein. Even the thiosulfonic parent compounds **9** and **10**[17] lower inhibited STAT3 compared to their corresponding drug hybrids, possibly for their higher hydrophilicity or too small size. Indeed, small methanethiosulfonate derivatives have been previously used to examine the accessibility of cysteine residues in ion channel and receptor proteins[39–42], because these reagents can react with the free thiol group of cysteine residues to form a mixed disulfide between the cysteine sulfur and the electrophilic moiety of the reagent. Of course, if the cysteine residue is in a critical region, the additional



Scheme 2. Reagents and conditions: (a) anh. DMF, N_2, 60 °C, 4 h.



Scheme 3. Reagents and conditions: (a) acetone, rt, 20 h; 80%. (b) HOBt, EDC-HCl, DIPEA, anh. DMF, Ar, 0 °C to rt, 5 h; 6%.

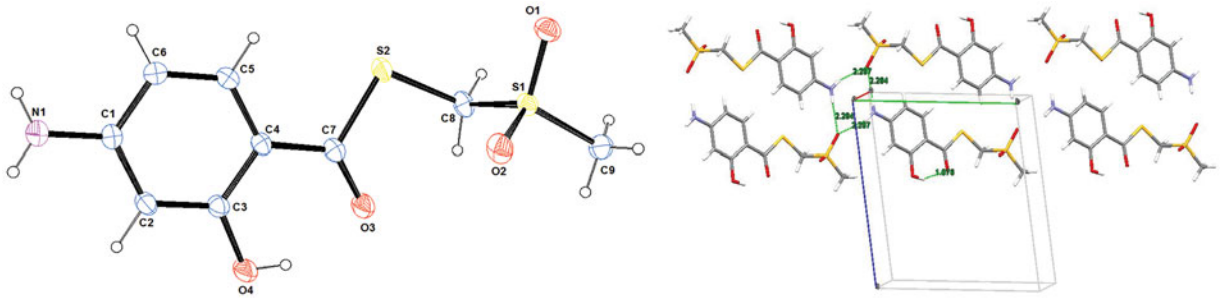


Figure 3. Left: ORTEP[34] drawing of **8**, showing the arbitrary atomic numbering (displacement ellipsoids at 40% probability). Right: Intermolecular interactions viewed about along *a*-axis.

Table 1. Comparative SH2 domain inhibitory activity and cytotoxicity on HCT-116 cell line of the tested compounds.

	STAT3			STAT1			Grb2	HCT-116
	% Inhibition			% Inhibition			% Inhibition	
Compound	30 μM	3 μM	IC_{50} (μM) ± SD	30 μM	3 μM	IC_{50} (μM) ± SD	3 μM	IC_{50} (μM) ± SD
1	101.7[a]	101.2 ± 2.2	0.7 ± 0.1	n.t.	44.9 ± 10.5	>3	76.0[a]	135.5 ± 2.2
2	105.8[a]	67.2 ± 11.0	1.4 ± 0.1	n.t.	1.9 ± 2.2	>3	−23.3[a]	NA[b]
3	101.8[a]	105.2 ± 8.2	0.5 ± 0.1	n.t.	46.7 ± 1.1	>3	74.6[a]	104 ± 10.9
4	100.0 ± 0.1	93.5 ± 2.6	1.4 ± 0.1	99.8 ± 0.4	44.2 ± 0.8	3.6 ± 0.0	15.9[a]	87.9 ± 11.4
5	101.8[a]	110.5 ± 2.2	0.5 ± 0.2	n.t.	35.4 ± 2.1	>3	77.8[a]	NA
6	58.4 ± 1.1	46.0 ± 0.8	4.9 ± 0.4	39.6 ± 0.8	18.0 ± 0.2	>30	n.t.	NA
8	41.8 ± 0.9	n.t.	>30	n.t.	n.t.	n.t.	n.t.	26.3 ± 11.6
VA	0.2 ± 3.2	n.t.	>30	2.7 ± 3.0	n.t.	>30	n.t.	NA
Sulindac	4.2 ± 3.2	n.t.	>30	4.9 ± 2.0	n.t.	>30	n.t.	NA
ASA	3.8 ± 1.9	n.t.	>30	6.3 ± 1.7	n.t.	>30	n.t.	NA
Diclofenac sodium salt	4.5 ± 2.4	n.t.	>30	6.5 ± 2.1	n.t.	>30	n.t.	NA
9	11.6[a]	n.t.	>30	n.t.	n.t.	n.t.	n.t.	NA[b]
10	−10.2[a]	46.8 ± 10.1	>3	n.t.	14.5 ± 7.1	>3	12.3[a]	NA[b]
15	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	NA
S3I-301	7.2 ± 1.7	n.t.	>30	n.t.	n.t.	n.t.	n.t.	39.5 ± 7.7

n.t.: not tested; NA: not active up to 200 μM.
[a]Mean of two experiments.
[b]Not active at 100 μM.

mass prevents normal function of the protein. In our case, the different inhibitory activity of our compounds suggests that bulky and lipophilic molecules are required. However, docking studies should be performed on these methanthiosulfonate hybrids in order to deeply investigate their interaction with STAT3-SH2 domain.

Despite the excellent *in vitro* STAT3 inhibition of the thiosulfonate-drug hybrids, only three compounds, **1**, **3**, and **4** showed a moderate antiproliferative activity on HCT-116 cell line, whereas the other two potent compounds, **2** and **5**, were inefficacious at concentration up to 100–200 μM. Since the STAT3 inhibition has been tested in a cell-free assay, the low cytotoxicity of the tested compounds could be related to their physicochemical properties, such as poor solubility and chemical stability in the culture medium (time-related hydrolysis), cell permeation, and enzymatic hydrolysis inside cells. These latter should be deeply explored in further studies, together with the cytotoxicity against other cancer cell lines.

As far as the new **S3I-201** analog **6**, the AlphaScreen results showed that it better interacts with the STAT3-SH2 domain compared to the parent compound (58.4% versus 7.2% of inhibition at 30 μM). Once again, this finding indicates the ability of the thiosulfonate moiety to bind the STAT3 SH2-domain. However, compound **6** did not exhibit any cytotoxic effect up to 200 μM, suggesting that it does not reach the target in whole cells, maybe for its low stability or for permeability reasons, which should be better explored.

Interestingly, compound **8**, characterized by an unusual sulfurated functionality, moderately inhibited STAT3-SH2 domain at 30 μM and exhibited a good cytotoxic activity on HCT-116 cell line which is comparable to that of **S3I-201** and is worthy of further investigation.

Conclusions

A set of thiosulfonic derivatives has been synthesized and tested *in vitro* by means of an AlphaScreen-based assay to investigate their ability to interact with STAT3-SH2 domain. Among the tested compounds, all the methanthiosulfonate drug hybrids were able to strongly and selectively bind STAT3-SH2 domain, with IC_{50} in low micromolar or submicromolar range, whereas the parent drugs were completely devoid of this ability. However, only few compounds showed a moderate cytotoxic activity on HCT-116 cell line, suggesting that, probably, these compounds do not reach easily their intracellular target due to unsuitable physicochemical properties. Therefore, further studies on chemical stability in the culture medium, as well as on solubility and cell permeability, are needed.

The obtained results suggest that the methanethiosulfonate moiety represents a useful scaffold for the synthesis of new direct STAT3 inhibitors and the methanethiosulfonate drug hybrids described in this manuscript can be considered interesting hit compounds worthy of structural optimization.

Worth of note is the occasional isolation of a compound with an unusual sulfurated functionality (**8**) and endowed with valuable antiproliferative activity on HCT-116 cell line, deserving further investigation.

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Disclosure statement

Prof. A. Sparatore is a coauthor of two patent applications describing two molecules studied in the manuscript. However these two patents have been abandoned several years ago and therefore she has no conflicts of interest to disclose.

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ORCID

Anna Sparatore http://orcid.org/0000-0003-2135-2649

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