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Synthesis and biological activity of diisothiocyanate-derived mercapturic acids

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ABSTRACT

This Letter deals with new non-natural diisothiocyanates, their mercapturic acid derivatives—conjugated with *N*-acetylcysteine as well as their antiproliferative activity towards human colon cancer cell lines and their inhibitory potency towards histone deacetylase activity. The activity of analysed isothiocyanates is not significantly different than their *N*-acetylcysteine conjugates. In comparison to simple mono-isothiocyanate analogues, aliphatic diisothiocyanates and their conjugates are much more active than the simple presence of two isothiocyanate functionalities could indicate.

Natural compounds are a convenient starting material for the development of new biologically active substances. One of the extensively studied groups of natural compounds, known as isothiocyanates (ITCs), possess chemoprotective properties and anti-carcinogenic activity. These low molecular weight organic compounds, stored in the form of glucosinolates, are present in many edible plants, particularly from the *Cruciferae* family, which include broccoli, cauliflower, cabbage, mustard or horseradish.[1] Multiple studies have demonstrated the chemopreventive effects of ITCs against chemical tumorgenesis in animal models where inhibition of carcinoma progression was observed in liver, bladder, colon, lung, mammary gland, prostate and pancreas.[1,2] Mechanisms involved in chemoprevention include the ability of ITCs to reduce the activation of carcinogens by inhibition of cytochrome P450 monooxygenases (phase I enzymes) as well as detoxification via activation of phase II enzymes (e.g., glutathione *S*-transferase, quinone reductase, glucuronyltransferase). Thus modulation of phase I and II enzymes activity brings about a protective effect against carcinogens induced damage of cell DNA.[3,4] Moreover, ITCs have an impact on multiple pathways including apoptosis, cell cycle arrest, angiogenesis and metastasis.[1]

Due to its electrophilic character, ITCs easily react with cellular thiols forming dithiocarbamates. The most important substrate for this reaction is glutathione (GSH). The ITC–GSH conjugation induces a further uptake of ITCs and its intracellular accumulation which can reach micromolar concentration.[5] Subsequently, the conjugate is rapidly depleted from the cell leading to the accumulation of reactive oxygen species (ROS) which consequently may induce cell apoptosis. This mechanism together with protein *S*- and *N*-thiocarbamoylation is believed to contribute to the anticancer activity of ITCs. The effluxed ITC–GSH conjugates are further metabolized forming mercapturic acid derivatives which are excreted into urine.[6] Alternatively, *S*-linked conjugates of isothiocyanates may undergo spontaneous hydrolysis in vivo, back to ITCs, and subsequently re-enter the cell.[7] The reversible character of isothiocyanate metabolism may explain the anticancer activity of *N*-acetylcysteine (NAC) conjugates of isothiocyanates which are the final product of ITC–GSH metabolism. Studies on prostate cancer cell lines have demonstrated that NAC conjugates of sulforaphane (SFN) show a similar activity as SFN itself on cell growth inhibition and apoptosis.[8] Moreover, NAC conjugates of ITCs inhibited lung tumorgenesis in the animal model[9] suggesting that *S*-linked conjugates of ITCs can serve as the latent, transporting form of parent isothiocyanates.

The accumulation of ROS as a result of ITCs treatment, which is observed in cancer cells but not normal cells, plays an important role in isothiocyanate-induced apoptosis. The process of ROS-mediated apoptosis involves several molecular mechanisms including activation of c-Jun-N-terminal kinases, Bax, caspases

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and inhibition of the mitochondrial respiratory chain or NF-κB activity.[2] In addition to the wide range of molecular targets associated with the antiproliferative activity of ITCs, the NAC and Cys-conjugates of isothiocyanates show inhibitory activity towards histone deacetylase (HDAC). In many cancer cells increased HDAC activity leads to dysregulation of the cell proliferation mechanisms. Inhibition of HDAC results in an enhanced histone acetylation and subsequently the activation of genes important for cell cycle control and apoptosis (e.g., *p21*, *bax*). The observed in vitro effects of HDAC inhibition are cell cycle arrest in G2/M phase and apoptosis, which coincides with the cellular response to ITCs.[10]

Limited bioavailability and structure-dependent activity of natural ITCs[6] are the catalyst for the development of new, synthetic isothiocyanates, with several examples displaying anticancer activity at a level similar to the natural products. Amongst the analysed compounds, the phenethyl isothiocyanate (PEITC) analogue—3,4-methyelendioxybenzyl isothiocyanate displayed a 6-fold increase in the NF-κB inhibitory activity when compared to the parent compound.[11] Similarly, 2,2-diphenylethyl ITC induces the apoptosis of breast cancer cells to a greater extent than natural benzyl isothiocyanate (BITC).[12] In our previous study we described a series of new, structurally diverse ITCs bearing dialkoxyphosphoryl functionality with antiproliferative activity comparable to PEITC and BITC.[13] In general, the previous results suggest that there is no distinct structure–activity relationship of isothiocyanates that may facilitate anticancer potency; however, the presence of the second functional group (e.g., polar, aromatic) seems to be important for the antiproliferative activity of ITCs.[14]

The main goal of the present study was to investigate the antiproliferative activity of a series of structurally diverse diisothiocyanate mercapturic acids towards cancer cells. Additionally, we have analysed the structure–activity relationship for obtained compounds in comparison with the selected mono-isothiocyanate mercapturic acids as well as non-conjugated mono- and diisothiocyanates, including naturally occurring isothiocyanates—BITC and PEITC. The antiproliferative activity was evaluated on the human colon adenocarcinoma cell line (LoVo) and the doxorubicin-resistant human colon adenocarcinoma cell line (LoVo/DX) by means of the sulforhodamine B (SRB) assay. The impact of the compounds under study on cells viability is presented as a 50% growth inhibitory concentration (IC_{50}) with doxorubicin used as reference. As a result of the fact that histone deacetylases are an emerging target for new chemotherapeutics, and isothiocyanate metabolites (mainly Cys- and NAC-conjugates of ITC) have shown inhibitory activity towards HDAC, the present study includes an analysis of the reported diisothiocyanate mercapturic acids potential to inhibit HDAC activity. The measurements were performed in the LoVo/DX cell lysate, used as a source of crude enzyme, by the indirect spectrofluorometric assay.[15,16] The synthetic protocols were based on known methods of synthesis of isothiocyanates[17] and mercapturic acids[18] and were described in detail in the Supplementary material along with the description of the biological experiments and the full characterisation of resultant compounds.

Since the relationship between the structure of ITCs and their biological activity is not straightforward, the designed series of 11 NAC-conjugates of diisothiocyanates (Table 1, compounds **1**–**11**) included structures bearing aliphatic, cyclic as well as aromatic groups. The antiproliferative activity has been observed in the micromolar range for both tested cell lines. The most promising group among obtained compounds were aliphatic analogues of diisothiocyanates with 1,4-butanedithiocarbamoyl-mercapturate (**2**) being the most active compound towards the LoVo cell line (IC_{50} = 2.02 μM) and one of the most potent towards LoVo/DX (IC_{50} = 4.84 μM). Interestingly, the *n*-butyl chain of compound **2** corresponds to the length of the alkyl chain in sulforaphane—probably the most intensively studied natural ITC, mainly due to its high and diverse biological activity. The analysis of several, previously evaluated, structural analogues of SFN indicates that its anticancer potency strongly depends on the presence of a second, polar group in the structure.[14] Such structural requirements may explain a dramatic loss of activity of compound **13**—mono-NAC-ITC conjugate bearing the unsubstituted *n*-butyl side chain. Its antiproliferative activity was over 64-fold lower than the one observed for compound **2** in the LoVo cell line (with an IC_{50} value of 130 μM) and more than 27-fold lower in the LoVo/DX cell line (IC_{50} = 134 μM). Moreover, similar activity was observed for compound **12** and to a lesser extent for compound **14**, both representing simple, aliphatic NAC derivatives of mono-ITCs. Activity that was similar to the one obtained for compound **2** towards the LoVo cell line, was observed for compound **5** with an IC_{50} value of 2.59 μM; however, the inhibition of the viability in the LoVo/DX cell line was over 1.6-times lower (IC_{50} = 7.93 μM) than in the case of compound **2**. The significant difference between *n*-butyl and the long *trioxa* moiety connecting thiocarbamoyl-mercapturate groups of compounds **2** and **5**, respectively, indicate that there is no direct correlation between the activity of the analysed NAC derivatives of diisothiocyanates and the length of the alkyl 'spacer' connecting thiocarbamoyl groups, especially considering the decrease in antiproliferative activity observed for analogues with the intermediate length of the aliphatic linker (compounds **3** and **4**). Nevertheless, the IC_{50} values of all analysed, aliphatic diisothiocyanate mercapturic acids were lower than 5 μM as evaluated in the LoVo cell line, and below 15 μM for the LoVo/DX cell line.

More significant differences in antiproliferative activity were observed for cyclic representatives of NAC-conjugates of diisothiocyanates with the most potent being *trans*-1,4-cyclohexanedithiocarbamoyl-mercapturate (**7**). The IC_{50} value for inhibition of LoVo cells viability was 5.21 μM, nevertheless compound **7** was the most active NAC conjugate towards the LoVo/DX cell line with an IC_{50} value of 3.45 μM. The shift in the thiocarbamoyl-mercapturate groups substitution in the cyclohexane ring from the 1,4-position in **7** to the 1,2-position in **6** resulted in an almost complete loss of activity. Interestingly, the close analogue of compound **7** with a methylsulfoxide substituent in the position occupied by the second thiocarbamoyl-mercapturate group was described by Posner and co-workers as an NAD(P)H quinone oxidoreductase 1 inducer with only a 2-fold decrease in activity in comparison to SFN, whereas unsubstituted cyclohexane-ITC showed a 280-times lower activity than that of SFN.[19] This example again indicates that the second, polar group incorporated into the isothiocyanate structure enables an increase in the anticancer activity of ITCs. However, compound **6** demonstrated that unsuitable positioning of such a substituent may significantly diminish its biological activity. The aromatic representatives of analysed NAC-conjugates of diisothiocyanates (compounds **9**–**11**) showed a moderate antiproliferative activity with the IC_{50} values being above 20 μM.

The introduction of an aromatic ring in compound **9** instead of a cyclohexane ring (**7**) resulted in the considerable loss of activity: 4.5-fold for the LoVo and 5.6-fold for LoVo/DX cell line. Moreover, in contrast to aliphatic diisothiocyanate mercapturic acids, NAC derivatives of the diisothiocyanate bearing aromatic group showed a similar or lower impact on cancer cells viability than aromatic mono-NAC–ITC conjugates. The IC_{50} values for BITC (**15**) and PEITC (**16**) NAC-conjugates were, respectively, 11.7 μM and 12.7 μM in the LoVo and 16.8 μM and 7.7 μM in the LoVo/DX cell lines. However, *p*-methoxybenzyl-thiocarbamoyl-marcapturate (**17**) was even more active than natural ITCs with IC_{50} values of 3.7 μM (LoVo) and 4.2 μM (LoVo/DX). An observed improvement in antiproliferative activity, in comparison with NAC-BITC, was attributed to the presence of a polar substituent in the *para* position of the aromatic ring.

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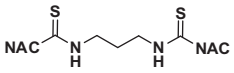
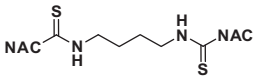
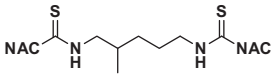
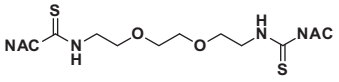
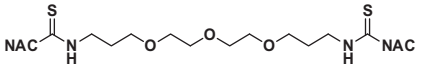
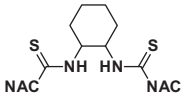
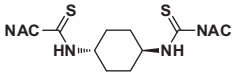
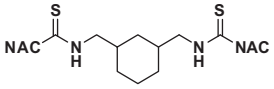
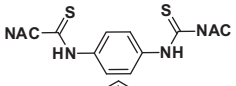
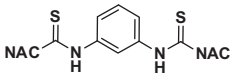
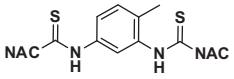
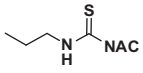
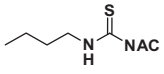
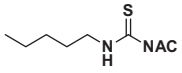
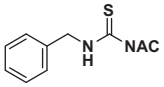
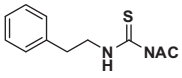
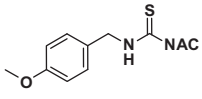
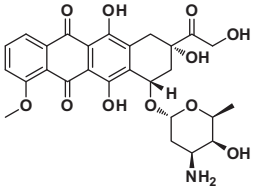
Table 1
Antiproliferative activity of mercapturic acid derivatives of diisothiocyanates, isothiocyanates and doxorubicin on LoVo and LoVo/DX cell lines and inhibition of histone deacetylase

	Compound	IC_{50}		
		LoVo[a] [μM]	LoVo/DX[a] [μM]	HDAC[b,c] [mM]
1		4.22 ± 1.5	9.55 ± 0.6	5.96 ± 0.07
2		2.02 ± 0.8	4.84 ± 2.5	2.33 ± 0.03
3		7.15 ± 2.7	6.60 ± 1.5	2.19 ± 0.04
4		4.62 ± 1.2	14.18 ± 2.9	4.37 ± 0.11
5		2.59 ± 0.7	7.93 ± 2.6	4.26 ± 0.03
6		NI	473.41 ± 87	1.66 ± 0.05
7		5.21 ± 2.4	3.45 ± 1.9	3.74 ± 0.09
8		11.66 ± 1.1	12.85 ± 2.1	2.68 ± 0.14
9		23.47 ± 10.44	19.38 ± 13.1	0.29 ± 0.01
10		>25	>25	0.97 ± 0.02
11		20.49 ± 10.84	22.35 ± 9.56	0.45 ± 0.01
12		150.8 ± 19	112 ± 16	ND
13		130 ± 11	134 ± 23	3.46 ± 0.06
14		36.6 ± 6	42.8 ± 2.5	3.20 ± 0.04
15		11.7 ± 0.9	16.8 ± 0.7	3.44 ± 0.02
16		12.7 ± 0.5	7.7 ± 0.3	3.45 ± 0.07
17		3.7 ± 0.5	4.2 ± 0.8	2.94 ± 0.08
Doxorubicin		0.15 ± 0.024	7.2 ± 1.1	ND

ND—not determined; NI—no inhibition; NAC = -$SCH_2C(NHAc)CO_2H$.
[a] IC_{50} values represent mean values ± SD from at least three experiments performed in triplicate.
[b] Determined on LoVo/DX cell lysates.
[c] IC_{50} values represent mean values ± SD from experiment performed in triplicate.

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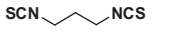
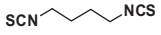
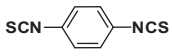
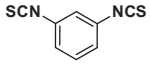
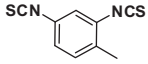
The antiproliferative activity of all synthesized mercapturic acids against normal mice fibroblasts cell line (Balb/3T3) was analysed. Almost all tested compounds showed activity comparable with the results obtained for the LoVo and LoVo/DX cell lines (Table S1). The most significant differences were observed for compounds **9** and **11**, where IC_{50} values in the Balb/3T3 cell line were approximately 3-times higher than in the LoVo or LoVo/DX cell lines. In contrast, IC_{50} values in Balb/3T3 for compounds **4** and **8** were almost 2-times lower in comparison to LoVo/DX cell line. It might suggest potential high toxicity of these compounds. However, it should be noted that IC_{50} values for cisplatin and doxorubicin (cytostatics used as positive control) displayed similar characteristics when tested in all three cell lines. Moreover, we did not observe distinctive differences in antiproliferative activity towards normal and malignant cell lines for NAC-conjugates of natural isothiocyanates (compounds **15** and **16**). Nevertheless, our preliminary in vivo toxicity study for compound **2** performed in Balb/c mice showed no toxic effects (based on body weight changes observation, blood morphology and blood biochemical analysis) when the compound was administered in high doses (900 μmol/kg b.w. [448.2 mg/kg b.w.]).

To study the activity of mercapturic acid analogues here reported in comparison with parent ITCs, we have analysed the antiproliferative activity of several isothiocyanates and diisothiocyanates on LoVo and LoVo/DX cell lines. The results (Table 2) show that there is no general increase in the activity in the ITCs group, and the IC_{50} values obtained for ITCs and their direct NAC-ITC analogues did not differ more than four-times from each other. Among the analysed ITCs the most potent inhibitors of cancer cells viability were 1,3-propane diisothiocyanate (**18**) and 1,4-butane diisothiocyanate (**19**). Both compounds were more active than their mercapturic acids analogues with the IC_{50} values of 1.84 μM (**18**) and 1.88 μM (**19**) in the LoVo, and 2.44 μM (**18**) and 2.35 μM (**19**) in LoVo/DX cell line. Similarly, as their NAC-ITC analogues, aliphatic isothiocyanates (compounds **23**–**25**) display a dramatic loss of activity in comparison to their diisothiocyanate analogues (**18**, **19**). Aromatic ITCs presented a moderate antiproliferative potency with the most active compound **28**, which showed the IC_{50} value of 3.7 μM in the LoVo cell line and 3.9 μM in the LoVo/DX cell line. The antiproliferative potency of synthesized diisothiocyanates and their mercapturic acid analogues towards the LoVo cell line, with the IC_{50} values near 2 μM for the most active compounds (**2**, **18**, **19**), is much lower than observed for doxorubicin with an IC_{50} value of 0.15 μM. However, the activity of several ITCs and NAC–ITCs conjugates tested towards the doxorubicin-resistant colon cancer cell line showed an increase in the activity when compared with doxorubicin (IC_{50} = 7.2 μM), with the most potent (**19**) being 3-times as active as doxorubicin. More importantly, the inhibitory potency of analysed compounds towards the LoVo cell line is comparable or moderately lower than the results obtained for the LoVo/DX cell line whereas the activity of doxorubicin was 48-times lower towards LoVo/DX cell line when compared with LoVo cells.

Table 2
Antiproliferative activity of diisothiocyanates and isothiocyanates on LoVo and LoVo/DX cell lines

	Compound	IC_{50} [μM]	
		LoVo[a]	LoVo/DX[a]
18		1.84 ± 0.4	2.44 ± 0.8
19		1.88 ± 0.3	2.35 ± 0.35
20		9.41 ± 1	11.6 ± 1.8
21		46.37 ± 7.32	55.72 ± 1.89
22		35.22 ± 13.10	47.32 ± 5.0
23		181 ± 29	177 ± 29
24		296 ± 41	265 ± 30
25		88.3 ± 7	75.2 ± 15
26		14.5 ± 2	19.8 ± 4
27		13.5 ± 1	9.8 ± 2
28		3.7 ± 0.6	3.9 ± 0.7

[a] IC_{50} values represent mean values ± SD from at least three experiments performed in triplicate.

The inhibitory activity of the obtained NAC-ITCs conjugates towards histone deacetylase was measured using LoVo/DX cells lysate. Contrary to the results from the proliferation assay, the most potent group of HDAC inhibitors were phenylene substituted diisothiocyanate mercapturic acids (compounds **9**–**11**) with an IC_{50} = 0.29 mM for the most active compound (**9**). Interestingly, considerably lower activity was observed for the majority of the remaining compounds, regardless of their structure (aromatic, aliphatic), including mono- and diisothiocyanate mercapturic acids, with compound **6** (IC_{50} = 1.66 mM) and compounds **2** and **3** (2.33 mM and 2.13 mM, respectively) being the most active HDAC inhibitors among them. Additionally, *N*-acetylcysteine alone displayed a similar inhibitory activity towards HDAC with an IC_{50} value of 3.16 mM. This suggests that in some cases the observed HDAC inhibition may be a result of NAC-ITC - enzyme binding and/or inhibitory activity of NAC alone, after it has been released from the NAC–ITC conjugates. Generally, it does not seem that HDAC inhibition contributed significantly to the overall activity of reported derivatives. However, it needs to be taken under consideration that isothiocyanates accumulate rapidly inside cancer cells reaching even millimolar concentrations.[20]

Multiple studies have demonstrated the impact of an additional substituent in isothiocyanates on their biological activity.[14] Nevertheless, there is only one report describing the anticancer activity of diisothiocyanates, with the structures based on the naphthalene diimide scaffold.[21] In the present study we analysed the antiproliferative activity and HDAC inhibitory potency for the series of structurally diverse diisothiocyanates and diisothiocyanate-derived mercapturic acids. The results revealed a significant increase in antiproliferative potency for aliphatic diisothiocyanate mercapturic acids as compared with NAC-ITCs bearing a single –N=C=S group. The definite differences in IC_{50} values between both groups suggest that the increased potency of diisothiocyanate mercapturic acids is not just a combined effect of the reactivity of the two isothiocyanate groups but may indicate different mechanisms of action or/and a significant increase in cellular uptake. Mercapturic acids are the final product of in vivo metabolism of isothiocyanate; however, the spontaneous hydrolysis enables their inversion to ITCs. The obtained NAC-ITC conjugates of synthetic as well as natural ITCs show a similar antiproliferative potency towards LoVo and LoVo/DX cell lines as parent ITCs. The cellular uptake of ITCs is probably facilitated by diffusion[4] and therefore the difference in activity between ITCs and corresponding mercapturic acids is the result of ITC potency but also the extracellular equilibrium between free ITC and its NAC-conjugate. Therefore, mercapturic acids as an odourless, mainly solid, latent form of ITCs present an attractive solution to the ITCs formulation for oral administration in future clinical use.

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Supplementary data

Supplementary data (synthetic and experimental procedures together with the characterisation of all obtained compound) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.11.045.

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