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Original article

Synthesis and antiproliferative activity of benzo[*d*]isothiazole hydrazones

Paola Vicini [a,*], Matteo Incerti [a], Irini A. Doytchinova [b], Paolo La Colla [c,*], Bernadetta Busonera [c], Roberta Loddo [c]

[a] *Dipartimento Farmaceutico Università degli Studi Parma, Parco Area delle Scienze 27/A–43100 Parma, Italy*
[b] *Faculty of Pharmacy, Medical University, 2 Dunav St., 1000 Sofia, Bulgaria*
[c] *Dipartimento di Scienze e Tecnologie Biomediche, Sez. Microbiologia e Virologia Generale e Biotecnologie Microbiche, Università di Cagliari, Cittadella Universitaria di Monserrato, 09042 Monserrato (CA) Italy*

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Abstract

Several benzo[*d*]isothiazole hydrazones have been evaluated for their potential antiretroviral activity. Since a number of these compounds were found to be inactive against viruses, but showed cytotoxicity at micromolar concentrations against the human $CD4^+$ lymphocytes (MT-4) that were used to support HIV-1 growth, they were further tested for antiproliferative activity. The compounds resulted as being cytotoxic for MT-4 cells and new derivatives which were rationally designed and synthesized, were tested for antiproliferative activity against several leukaemia and solid tumour cell lines. In addition, these compounds were evaluated against "normal" cell lines. Compound **2h** proved to be the most active compound and the fragment –CO–NH–N=CH–2-hydroxyphenyl was identified as being very important for biological activity, suggesting intramolecular hydrogen bond formation or favourable mutual disposition between two important centres in the pharmacophore. ^{1}H-NMR spectra have been explained with the support of a conformational analysis.

Keywords: Synthesis; Benzo[*d*]isothiazole hydrazones; Conformational analysis; Cytotoxicity; Antiproliferative activity

1. Introduction

As a continuation of our studies on the chemistry and pharmacology of benzo[*d*]isothiazole derivatives [1–5], we have recently reported on the synthesis of benzo[*d*]isothiazole Schiff bases which revealed remarkable in vitro antileukaemic activity [6]. In our attempt to further investigate benzo[*d*]isothiazole derivatives as potential antineoplastic agents, we have been prompted to synthesise and test, as a rational development of molecules resulting as being cytotoxic against the lymphoid MT-4 cell line during an antiretroviral screening program (P. Vicini: manuscript in preparation), a new series of benzo[*d*]isothiazole hydrazones sharing an azomethine moiety with the benzo[*d*]isothiazole Schiff bases. The benzo[*d*]isothiazole hydrazones which are the subject of the present investigation belong to five groups of general structures **1–5** shown in Fig. 1.

Based on the fact that structure **2** emerged in our preliminary investigation as the most promising for antiproliferative activity, it was used as a scaffold and was subjected to structural modifications. In view of the favourable effect shown by the R hydroxy group in some of the early tested compounds (**2h**, **3h**, **5h**) we synthesized the unknown hydroxybenzylidene derivatives **2m**, **2n** and the 3,4-dihydroxy derivative **2r**, which can be regarded as the open analogue of the benzo[1,3]dioxol derivative **2k**, which also resulted as being highly active. In addition, we prepared compounds **2o, 2p** and **2q** by introducing in *ortho*, *meta* and *para* positions a fluorine atom as functional R substituent, with the awareness that the selective introduction of a fluorine atom, bioisoster of hydroxyl, could be an effective tool for modifying the physicochemical properties and, consequently, the biological behaviour of starting com-

* Corresponding authors.
E-mail addresses: pvicini@ipruniv.cce.unipr.it (P. Vicini), placolla@unica.it (P. La Colla).

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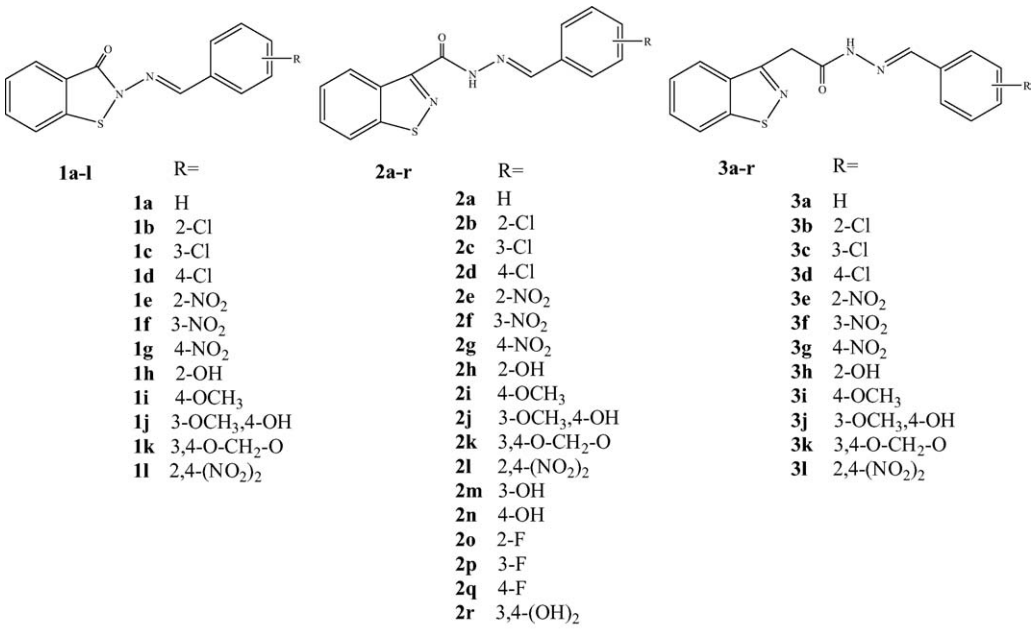

1a-l R=

1a H
1b 2-Cl
1c 3-Cl
1d 4-Cl
1e 2-NO_2
1f 3-NO_2
1g 4-NO_2
1h 2-OH
1i 4-OCH_3
1j 3-OCH_3,4-OH
1k 3,4-O-CH_2-O
1l 2,4-$(NO_2)_2$

2a-r R=

2a H
2b 2-Cl
2c 3-Cl
2d 4-Cl
2e 2-NO_2
2f 3-NO_2
2g 4-NO_2
2h 2-OH
2i 4-OCH_3
2j 3-OCH_3,4-OH
2k 3,4-O-CH_2-O
2l 2,4-$(NO_2)_2$
2m 3-OH
2n 4-OH
2o 2-F
2p 3-F
2q 4-F
2r 3,4-$(OH)_2$

3a-r R=

3a H
3b 2-Cl
3c 3-Cl
3d 4-Cl
3e 2-NO_2
3f 3-NO_2
3g 4-NO_2
3h 2-OH
3i 4-OCH_3
3j 3-OCH_3,4-OH
3k 3,4-O-CH_2-O
3l 2,4-$(NO_2)_2$

4a-l R=

4a H
4b 2-Cl
4c 3-Cl
4d 4-Cl
4e 2-NO_2
4f 3-NO_2
4g 4-NO_2
4h 2-OH
4i 4-OCH_3
4j 3-OCH_3,4-OH
4k 3,4-O-CH_2-O
4l 2,4-$(NO_2)_2$

5a-l R=

5a H
5b 2-Cl
5c 3-Cl
5d 4-Cl
5e 2-NO_2
5f 3-NO_2
5g 4-NO_2
5h 2-OH
5i 4-OCH_3
5j 3-OCH_3,4-OH
5k 3,4-O-CH_2-O
5l 2,4-$(NO_2)_2$

Fig. 1. Chemical structures of the benzo[*d*]isothiazole hydrazones under study.

pounds [7,8]. In addition, we synthesized compounds **1l, 2l, 3l, 4l** and **5l** in order to evaluate the effect of a 2,4-dinitrobenzylidene moiety on the five structures **1–5,** taking into account that nitro groups can improve the cytotoxicity.

The present paper describes the synthesis and the evaluation of the in vitro antiproliferative activity of benzo[*d*]isothiazole hydrazones **1l, 2h, 2i, 2k-2r, 3h**, **3l**, **4l**, **5h**, **5l** against a panel of human cell lines derived from haematological, solid tumours and normal cells. The structure–activity relationship is discussed in terms of potency and selectivity.

2. Chemistry

The target benzo[*d*]isothiazole hydrazones were prepared according to the general scheme shown in Fig. 2. Briefly, starting cyclic hydrazides **1** and **4** were prepared by cyclization of the suitable 2-chlorocarbonylphenylsulfenylchloride by treatment with *N*-BOC protected hydrazine, followed by mild acidic hydrolysis (trichloroacetic acid) for removal of the BOC protective group. Acyclic hydrazides **2**, **3** and **5** were alternatively prepared by reaction of hydrazine hydrate with the appropriate ester derived from carboxylic acid. Condensation of hydrazides **1**–**5** with the appropriate aldehydes yielded the hydrazide-hydrazones, in good amounts, in accordance with the previously reported general method [2,3].

The structures of the target compounds were characterized using UV, FT-IR and ^{1}H-NMR spectral analysis and by comparison with the available literature data. All new compounds gave satisfactory elemental analyses (C, H, N, S) within ± 0.4% of the theoretical values. Physical and spectral data are given in Table 1.

The IR spectra showed the azomethine C–H stretching double absorption band, of medium intensity, in the 2930–2730 cm^{-1} region. The above bands, accompanied by a C=N absorption band between 1550 and 1600 cm^{-1}, are good evidence for the presence of an azomethine linkage. An addi-

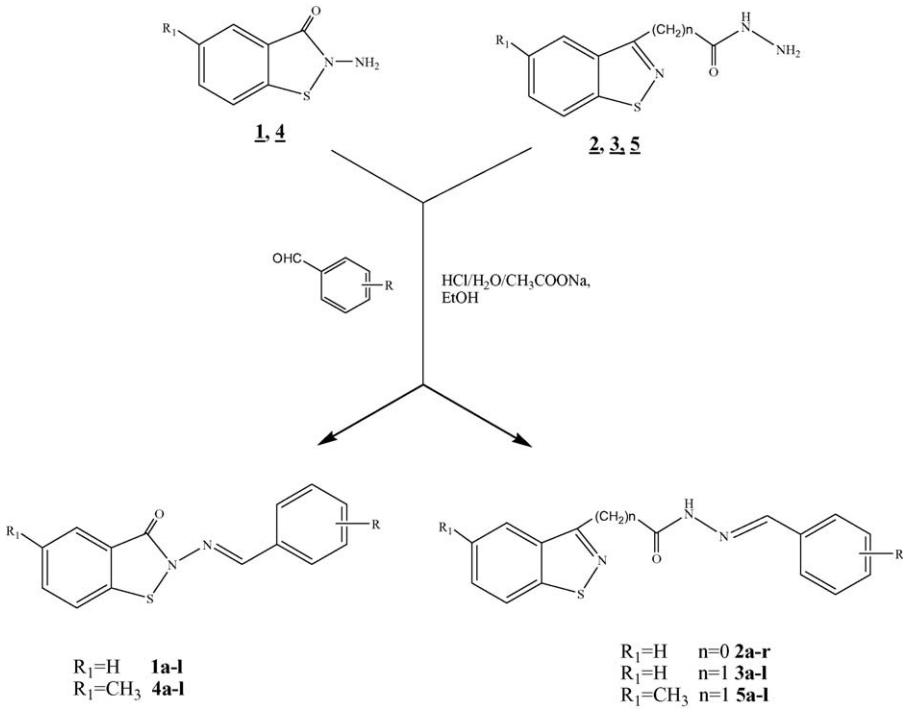

Fig. 2. Scheme of synthesis of benzo[*d*]isothiazole hydrazones.

tional strong band in the 1690–1640 cm^{-1} region, attributable to a carbonyl stretching, confirmed the hydrazono feature of all the compounds. The IR spectra of the benzo[*d*]isothiazole hydrazones **2**, **3** and **5** exhibited, in the 3330–3060 cm^{-1} range, the NH weak band of the CO–NH–N = function. The absorption bands associated with the R substituents appeared in the expected regions.

In the ^{1}H-NMR spectra of all the benzo[*d*]isothiazole hydrazones, the signal representing the azomethine CH protons appeared at 8.39–9.10 ppm, whereas **2**, **3** and **5** NH amidic protons (–CONHN=CH–) resonated at 10.21–12.54 ppm. It should be noted that, as already observed for all the benzo[*d*] isothiazole hydrazones of structure **3** and **5** [2], the ^{1}H-NMR spectra of new compounds **3l** and **5l**, at room temperature, besides the expected signals, also evidenced resonances attributable to the presence in DMSO-d_6 solution of an additional species (about 33%). In particular, **3l** and **5l** spectra show double signals corresponding to amidic NH proton, benzylidene moiety protons, methylene and methyl protons (Table 1). These data, in agreement with the existence of stable conformers for compounds **3l** and **5l**, are consistent with the spectral data registered at high temperature (353 K) that show, as expected, well resolved resonances attributable to a single conformational structure (data not shown). Moreover, some interesting observations can be made by comparing the ^{1}H-NMR spectra of the starting hydrazides 3 and 5 with the target compounds **3l** and **5l**: no conformational equilibrium, in the same experimental conditions, is observed for hydrazides **3** and **5**; the shift of the singlet of the amidic NH from about 9.41 in the intermediate hydrazide to $\delta = 11.10$ and $\delta = 12.40$, respectively, in **3l** and **5l** may be considered as proof of the formation of an intramolecular N….H bond [2]. The decreasing basicity following the benzylidene substitution in the intermediate compounds is therefore critical for the formation of stable conformers due to an N….H bond. This can be explained by an intramolecular H bonding interaction connecting the endocyclic N nitrogen and the NH group of the CONH side chain, favoured by a six-member ring system in derivatives **3** and **5**, but destabilized by a five-member ring system in **2**, the lower homologues of compounds **3**, which accordingly do not show any conformational equilibrium in ^{1}H-NMR spectra. To complete this study, a comparative conformational analysis was carried out for compounds **2l** and **3l,** aimed at confirming the hypothesis of a low-energy conformer stabilized by an intramolecular H bond only for the latter [9].

3. Results

3.1. Biological results

Several hydrazones of benzo[*d*]isothiazole hydrazides had been evaluated for their capability to inhibit HIV-1 multiplication in MT-4 cells-based assays (data not shown) (P. Vicini: manuscript in preparation). The compounds **2h**, **2i**, **2k**, **3h**, **5h** resulted as being devoid of antiretroviral activity although they showed cytotoxicity against MT-4 cells at micromolar concentrations. The above compounds and the new derivatives **1l, 2l**–**2r**, **3l**, **4l**, **5l**, rationally designed for the purpose of this study, were further tested for antiproliferative activity against a panel of human cell lines derived from both haematological (CCRF-CEM, WIL-2NS and CCRF-SB) and solid (SKMEL28, MCF7, SKMES-1, HepG2, and DU145) tumours, in order to evaluate their potential antitumour activity and to obtain some insights into their spectrum of activity (Tables 2 and 3). All

Table 1
Physical properties and spectral data of tested compounds

Compound	Molecular formula (MW)	m.p. (°C)	Yield (%)	UV (λ_{max}, nm)	IR (ν, cm^{-1})	^{1}H-NMR (δ, ppm)
1l	$C_{14}H_8N_4O_5S$ (344.30)	243–245 dec	85	391 298 245 206	2922, 2839; 1683; 1596	8.82 (s, 1H, H-3'); 8.66–8.62 (m, 2H, H-5', CH); 8.36 (d, 1H, *J* = 8.4, H-6'); 8.09–8.01 (m, 2H, H-4, H-7); 7.82 (t, 1H, *J* = 7.5, H-5); 7.53 (t, 1H, *J* = 7.5, H-6)
2h	$C_{15}H_{11}N_3O_2S$ (297.33)	268–270[a]	90	341 270 216	[2]	[2]
2i	$C_{16}H_{13}N_3O_2S$ (311.36)	183–185	95	357 338 251 215	[2]	[2]
2k	$C_{16}H_{11}N_3O_3S$ (325.34)	235–237	98	360 339 215	[2]	[2]
2l	$C_{15}H_9N_5O_5S$ (371.33)	283–285[a]	97	331 241 212	3160; 2929, 2867; 1670; 1598	10.21 (s, 1H, NH); 9.10 (s, 1H, CH); 8.80 (s, 1H, H-3'); 8.74 (d, 1H, H-6'); 8.60 (d, 1H, H-5'); 8.40 (d, 1H, *J* = 8.1, H-4); 8.34 (d, 1H, *J* = 8.1, H-7); 7.74–7.62 (m, 2H, H-5, H-6)
2m	$C_{15}H_{11}N_3O_2S$ (297.33)	215–216	93	321 270 245 218	3257; 2847, 2749; 1641; 1577	12.30 (s, 1H, NH); 9.67 (s, 1H, OH); 8.74 (d, 1H, *J* = 8.1 4); 8.50 (s, 1H, CH); 8.33 d, 1H, *J* = 8.4, H-7); 7.72–7,60 (m, 2H, H-5, H-6); 7.29–7.21 (m, 2H, H-5', H-2'); 7.10 (d, 1H, *J* = 7, H-6'); 6.84 (d, 1H, *J* = 7.5, H-4')
2n	$C_{15}H_{11}N_3O_2S$ (297.33)	284–286	85	327 274 217	3141; 2805, 2758; 1650; 1592	12.14 (s, 1H, NH); 9.99 (s, 1H, OH); 8.72 (d, 1H, *J* = 8.1, H-4); 8.47 (s, 1H, CH); 8.32(d, 1H, *J* = 8.1, 7); 7.72–7.56 (m, 4H, H-5, H-6, H-2', H-6'); 6.84 (d, 2H, *J* = 8.1, H-3', H-5')
2o	$C_{15}H_{10}FN_3OS$ (299.32)	234–235	98	322 306 268 212	3162; 2848, 2780; 1658; 1544	12.54 (s, 1H, NH); 8.66 (s, 1H, CH); 8.74 (d, 1H, *J* = 8.1, H-4); 8.33 (d, 1H, *J* = 8.4, H-7); 7.98 (t, 1H, *J* = 7.5, H-5); 7.71 (t, 1H, *J* = 6.9, H-5'); 7.64 (t, 1H, *J* = 7.8, H-6); 7.55–7.48 (m, 1H, H-6'); 7.34–7.28 (m, 2H, H-4', H-3')
2p	$C_{15}H_{10}FN_3OS$ (299.32)	191–192	97	323 306 270 212	3226; 2852, 2729; 1666; 1598	12.48 (s, 1H, NH); 8.73 (d, 1H, *J* = 8.1, H-4); 8.60 (s, 1H, CH); 8.34 (d, 1H, *J* = 8.1, H-7); 7.33–7.49 (m, 5H, H-5, H-6, H-2', H-6', H-5'); 7.33–7.28 (m, 1H, H-4')
2q	$C_{15}H_{10}FN_3OS$ (299.32)	157–158	87	325 305 269 216	3203; 2841, 2746; 1671; 1542	10.88 (s, 1H, NH); 8.73 (d, 1H, *J* = 8.1, H-4); 8.59 (s, 1H, CH); 8.32 (d, 1H, *J* = 8.1, H-7); 7.83–7.78 (m, 2H, H-2', H-6'); 7.73–7.60 (m, 2H, H-5, H-6); 7.34–7.29 (m, 2H, H-3', H-5')
2r	$C_{15}H_{11}N_3O_3S$ (313.33)	288–289	90	336 274 238 217	3243; 2854, 2734; 1644; 1587	12.09 (s, 1H, NH); 9.45 (s, 1H, OH); 9.32 (s, 1H, OH); 8.73 (d, 1H, *J* = 8.1, H-4); 8.39 (s, 1H, CH); 8.32 (d, 1H, *J* = 8.1, H-7); 7.72–7.60 (m, 2H, H-5, H-6); 7.25 (s, 1H, H-2'); 6.92 (d, 1H, *J* = 8.1, H-6'); 6.78 (d, 1H, *J* = 7.8, H-5')
3h	$C_{16}H_{13}N_3O_2S$ (311.36)	220–222[a]	93	320 278 218	[2]	[2]
3l	$C_{16}H_{11}N_5O_5S$ (385.36)	170–171	96	340 303 224 210	3185; 2916, 2866; 1689; 1604	11.11 and 10.82 (2 s, 1H, NH); 8.78 and 8.75 (2 d, 1H, *J* = 1.8, H-3'); 8.72 and 8.45 (2 s, 1H, CH); 8.57 and 8.40 (2 dd, 1H, *J* = 2.1, *J* = 9, H-5'); 8.32 (d, 1H, *J* = 9, H-6'); 8.21–8.13 (m, 3H, H-6', H-4, H-7); 7.63 (t, 1H, *J* = 6.9, H-5); 7.53 (t, 1H, *J* = 7.2, H-6); 4.58 and 4.23 (2s, 2H, CH_2).
4l	$C_{15}H_{10}N_4O_5S$ (358.33)	257–259[a]	80	395 305 247 206	2916, 2852; 1700; 1596	8.82 (d, 1H, *J* = 2.4, H-3'); 8.66–8.62 (m, 2H, H-5', CH); 8.38 (d, 1H, *J* = 8.7, H-6'); 7.94 (d, 1H, *J* = 8.1 H-6); 7.84 (s, 1H, H-4); 7.66 (dd, 1H, *J* = 1.2, *J* = 8.4, H-5)
5h	$C_{17}H_{15}N_3O_2S$ (325.39)	225–228[a]	97	318 267 216	[2]	[2]
5l	$C_{17}H_{13}N_5O_5S$ (399.38)	177–178	85	338 306 226 209	3100; 2922, 2839; 1671; 1598	12.40 and 12.12 (2 s, 1H, NH); 8.79 and 8.76 (2 d, 1H, *J* = 2.7, H-3'); 8.71 and 8.44 (2 s, 1H, CH); 8.58 and 8.41 (2 dd, 1H, *J* = 2.4, *J* = 9, H-5'); 8.32 and 8.18 (2 d, 1H, *J* = 9, H-6'); 8.08 (dd, 1H, *J* = 4.2, *J* = 8.4, H-6); 7.99 and 7.95 (2 s, 1H, H-4); 7.48 (d, 1H, *J* = 8.1, H-7); 4.54 and 4.19 (2 s, 2H, CH_2); 2.50 and 2.47 (2 s, 3H, CH_3).

[a] From 1,4-dioxane.

derivatives were also evaluated against "normal" cell lines (CCL-75, MRC-5, CRL7065, resting and proliferating PBL) (Table 4). 6-Mercapto-purine (6MP) was used as reference drug.

A number of title compounds inhibited the proliferation of different cell lines deriving from haematological tumours in the micromolar range. Generally, they were less active against normal cells and cells deriving from solid tumours. Among the new derivatives, none showed a spectrum of antiproliferative activity better than that of the first generation counterparts. Compound **2h**, bearing a hydroxy group at position 2 of the benzylidene moiety, resulted as being the most potent, showing

Table 2
Antiproliferative activity of compounds against leukaemia-/lymphoma-derived cell lines

Compound	IC_{50} (μM)[a]			
	MT4[b]	CCRF-CEM[c]	WIL-2NS[d]	CCRF-SB[e]
1l	85	66	> 100	> 100
2h	1.3	0.5	3.1	1.4
2i	3	12.9	9.9	4.3
2k	14	75	> 100	62
2l	> 100	> 100	> 100	> 100
2m	56	30	> 100	47
2n	> 100	74	> 100	73
2o	50	64	> 100	79
2p	25	21	76	24
2q	87	83	> 100	59
2r	9.2	9.9	18	13
3h	5	4	14	8
3l	15	16	22	16
4l	> 100	> 100	> 100	> 100
5h	4.4	3	9	6
5l	10	12	20	7
6MP	0.1	1	3	1

[a] Compound concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± S.D.) for three independent determinations.
[b] $CD4^+$ human T-cells containing an integrated HTLV-1 genome.
[c] $CD4^+$ human acute T-lymphoblastic leukaemia.
[d] Human splenic B-lymphoblastoid cells.
[e] Human acute B-lymphoblastic leukaemia.

Table 3
Activity of compounds against solid tumour-derived cell lines

Compound	IC_{50} (μM)[a]				
	SK-MEL-28[b]	MCF7[c]	SKMES-1[d]	HepG2[e]	DU145[f]
1l	41	80	67	> 100	90
2h	2.5	2.1	1.8	7.6	8
2i	> 20	11	> 20	n.d.[g]	> 20
2k	> 20	> 20	> 20	n.d.[g]	> 20
2l	> 100	> 100	> 100	> 100	> 100
2m	> 100	> 100	76	63	> 100
2n	> 100	> 100	28	> 100	> 100
2o	> 100	> 100	> 100	> 100	> 100
2p	> 100	> 100	> 100	83	> 100
2q	> 100	> 100	> 100	68	> 100
2r	47	50	29	66	66
3h	9	8.4	8.4	10	14
3l	33	25	17	25	23
4l	> 100	> 100	> 100	> 100	> 100
5h	6	8.7	5.8	7.8	9.4
5l	21	20	13	15	14
6MP	15	3	58	8	2

[a] Compound concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± S.D.) for three independent determinations.
[b] Human skin melanoma.
[c] Human breast adenocarcinoma.
[d] Human lung squamous carcinoma.
[e] Human hepatocellular carcinoma.
[f] Human prostate carcinoma.
[g] Not determined.

an IC_{50} against the various cell lines ranging between 0.5 and 8.0 μM, thus resulting as being equally as potent as 6-MP against the haematological tumours. It is interesting to note that the above compound resulted as being 6- to 32-fold more potent than 6-MP against skin melanoma and lung squamous carcinoma, and equally as potent as 6-MP against breast adenocarcinoma, hepatocellular and prostate carcinoma. The 2-hydroxy substituted derivatives **3h** and **5h** were the only compounds of the series **3** and **5** that showed activity at micromolar concentrations. The hydroxy substitution effect is position-connected and not additive, as is evident from the higher activities of the 2-hydroxyderivatives than the activities of 3- or/and 4-hydroxylphenyls. Thus the cytotoxicity of the hydroxy derivatives decreases in the following order: **2h > 5h > 3h > 2r > 2m > 2n.**

The substitution of the hydroxy group for fluorine yielded compounds that resulted as being marginally active or inactive against both haematological and solid tumour cells. Moreover, of the hydroxy substituted compounds, the *ortho* derivative **2h** was endowed with the best antiproliferative properties, whereas substitution with fluorine at the *m*-position in the phenyl ring (**2p**) was preferred for antiproliferative activity to substitutions at *o*- and *p*-positions (**2o** and **2q**).

The introduction of 2,4-dinitro substituent did not improve the antiproliferative activity displayed by the hydroxy unsubstituted analogues, but, in general, resulted as being more effective than the fluorine substitution. Consistently with the inactivity against MT-4 cells previously observed for compounds **1** and **4** (data not shown), the new derivatives **1l** and **4l** resulted as being devoid of significant cytotoxicity. It is worth noting that, in the case of the 2,4-dinitro substitution, only derivatives **5** and **3** resulted as being slightly active (**5l > 3l),** while compound **2l** surprisingly resulted as being non-cytotoxic. It is also worth noting that none of the 2,4-dinitro derivatives exhibited cytotoxicity against normal cell lines.

With respect to selectivity, the most active compounds, **2h**, **3h** and **5h,** showed almost the same level of antiproliferative potency against haematological, solid tumour-derived and normal cells. On the other hand, compounds **2i** and **2k** were less potent than the 2-hydroxy derivatives **2h**, **3h** and **5h,** but selectively cytotoxic against a few or all the leukaemia cell lines, being non-cytotoxic against solid and normal cells.

3.2. Conformational analysis

The results of conformational analysis are shown in Figs. 3 and 4. For **3l** the shortest distance between N8 (heterocycle N) and H (NH chain group) is 1.897 Å and it exists at torsions N8C7C10C27 3.7° and C7C10C27O25 177.8° (the pink circle on the conformational map). As is evident from the map, this conformer belongs to the preferred low-energy conformers. For **2l** the shortest distance between N8 and H (NH group) is 2.345 Å at torsion N8C7C27O25 179.9°. Because of the p-π conjugation **2l** has a planar structure and no free rotation exists here. Only two conformers are possible — with torsions N8C7C27O25 0° and 180°. As a hydrogen bond may be formed if the distance between the H-atom and the H-bond acceptor atom is between 1.8 and 2.0 Å, our hypothesis as to the intramolecular H-bond of the compounds **3l** is confirmed and a favoured low-energy conformer, obtained by folding of

Table 4
Activity of compounds against "normal" cell lines

Compound	IC_{50} (μM)[a]					
	CRL7556[b]	CCL-75[c]	MRC-5[d]	CRL7065[e]	Resting PBL[f]	Proliferating PBL[g]
1l	n.d.[h]	> 100	> 100	> 100	> 100	> 100
2h	3	n.d.[h]	n.d.[h]	5 ± 1	n.d.[h]	n.d.[h]
2i	> 20	n.d.[h]	n.d.[h]	> 20	n.d.[h]	n.d.[h]
2k	> 20	n.d.[h]	n.d.[h]	> 20	n.d.[h]	n.d.[h]
2l	n.d.[h]	> 100	> 100	> 100	> 100	> 100
2m	n.d.[h]	90	84	> 100	> 100	80
2n	n.d.[h]	>100	> 100	> 100	> 100	> 100
2o	n.d.[h]	> 100	> 100	> 100	> 100	> 100
2p	n.d.[h]	> 100	77	> 100	> 100	> 100
2q	n.d.[h]	> 100	> 100	> 100	> 100	> 100
2r	n.d.[h]	70	58	> 100	> 100	74
3h	n.d.[h]	n.d.[h]	25	21	n.d.[h]	n.d.[h]
3l	n.d.[h]	> 100	> 100	> 100	> 100	> 100
4l	n.d.[h]	> 100	> 100	> 100	> 100	> 100
5h	n.d.[h]	n.d.[h]	18	13	n.d.[h]	n.d.[h]
5l	n.d.[h]	> 100	> 100	> 100	> 100	> 100
6MP	> 100	> 100	> 100	n.d.[h]	n.d.[h]	n.d.[h]

[a] Compound concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± S.D.) for three independent determinations.
[b] Ewing's sarcoma.
[c] Human lung fibroblasts.
[d] Human lung fibroblasts.
[e] Human foreskin fibroblasts.
[f] Resting peripheral blood lymphocytes.
[g] Proliferating peripheral blood lymphocytes.
[h] Not determined.

the 3- side chain over the aromatic heterocycle, in equilibrium with the unfolded isomer, is in agreement with that displayed by ^{1}H-NMR spectra.

4. Discussion and conclusions

The most potent antiproliferative activity was displayed by some benzo[*d*]isothiazole hydrazones **2.** No significant cytotoxicity against MT-4 cells was demonstrated (data not shown) by any of the derivatives with structures **1** and **4,** or by derivatives of types **3** and **5**, with the exception of 2-hydroxy derivatives **3h, 5h,** which demonstrated cytotoxicity at micromolar concentrations (respectively 5 and 4.4 μM).

This trend is well exemplified by comparing the results against MT-4 cells of the present and the previously tested benzo[*d*]isothiazole hydrazones of structures **1, 2, 3, 4** and **5,** bearing the same substituted benzylidene group: cytotoxicity dramatically fell when derivatives **2** were compared to the analogue counterparts in the following order **2 > 5 > 3> 1 = 4** (with the exception of **2l, 3l** and **5l;** cf. Biological results).

As regards the structure–activity relationship of series **2**, the benzo[*d*]isothiazole-3-carboxylic acid benzylidene hydrazides show antiproliferative activity which directly correlates with the polarity of the molecules − the more polar compounds are also the more active. These results indicate that the 2-hydroxybenzylidene group plays an important role in the biological interaction responsible for the activity under study. Thus, compound **2h** was the most potent of all the compounds tested; significant activity was also shown by **3h** and **5h**.

The results of this study indicate that in the benzo[*d*]isothiazole hydrazones the structural requirements both of the scaffold and of the benzylidene substitution are quite stringent. Thus the fragment –CO−NH−N=CH−2-hydroxyphenyl is very important for the activity, suggesting an intramolecular hydrogen bond formation or favourable mutual disposition between two important centres in the pharmacophore, the donor oxygen atom from the 2-hydroxy group and the acceptor oxygen atom from the carbonyl group. A third important centre in the pharmacophore is the amidic CONH group, as may be inferred from its presence in all the active derivatives and from its absence in the inactive compounds with structures **1** and **4.**

Several factors could account for these results. One is the profound difference in the conformational shape of the five series of derivatives. The **1** and **4** derivatives represent two flat structures connected by three flexible bonds [3], while **2** derivatives have a planar structure and no free rotation (cf. results of conformational analysis). On the other hand, in the series **3** and **5** the presence of a methylene bridge confers higher flexibility compared to **2**. This allows us to speculate that derivatives of series **2**, bearing favourable R substituents, could act with a cytostatic mechanism by intercalating with DNA as is well known for many planar polycycles [10–13], natural and synthetic DNA-intercalating agents. Also benzo[*d*]isothiazole hydrazones **3** and **5**, appropriately substituted, but not **1** and **4,** could cause antiproliferative activity through a presumably different mechanism of interaction with the biological target.

In conclusion, although last generation derivatives have not improved on the potency of their first generation counterparts, the development of the study by the evaluation of the cytotoxicity of benzo[*d*]isothiazole hydrazones against several cell lines gave some interesting insights into the potency and selec-

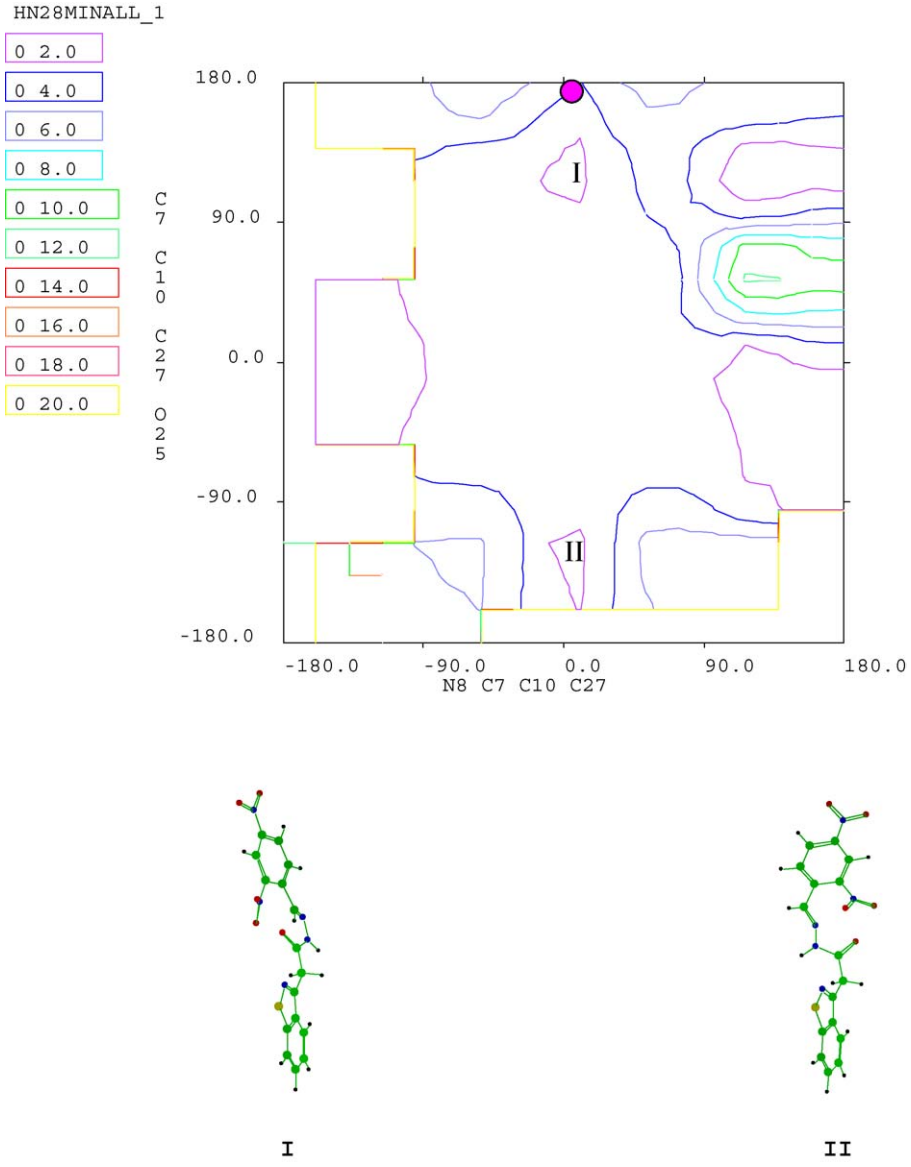

Fig. 3. A 2D conformational map of **3l** and low-energy conformers. Energy levels (in kcal mol^{-1}) are given in different colours. The pink circle shows the conformer with the shortest distance between N8 and H from NH group (Fig. 4, upper panel).

tivity of action. The overall results can reveal trends in the relationship between ligand structures and their antiproliferative activities in the class of benzo[*d*]isothiazole hydrazones, which may be a guide to future work in antitumour agent research and development. Further synthetic efforts aimed at improving the antiproliferative activity and experiments aimed at defining the target and the mechanism of the inhibitory effect shown by the molecules studied are in progress.

5. Experimental protocols

5.1. Chemistry

Melting points (°C) were determined with a Buchi 512 apparatus and are uncorrected. New compounds were analysed in the analytical laboratory of the Dipartimento Farmaceutico, Università di Parma, on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyser, for C, H, N and S. The values found were always ± 0.4% of the theoretical ones. UV spectra were recorded, in methanol, on a Jasco V-570 UV/VIS/NIR spectrophotometer (Jasco Ltd., Tokyo, Japan). UV absorption bands are given as λ_{max}, in nm. IR spectra, such as KBr pellets, were recorded on a Jasco FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan); wave numbers in the IR spectra are given in cm^{-1}. ^{1}H-NMR spectra of the newly synthesized compounds, in DMSO-d_6 solutions, were recorded on a Bruker AC 300 instrument at 298 K. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard; coupling constants *J* are expressed in Hertz. The reactions were followed by TLC on F_{254} silica-gel precoated sheets (Merck) and the purified compounds each showed a single spot. Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting

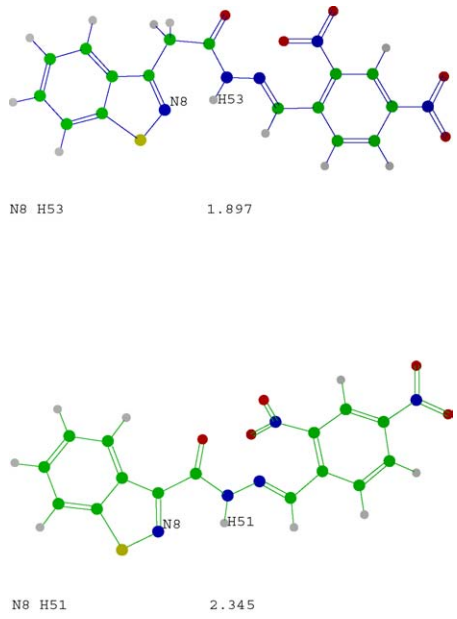

Fig. 4. H-bond formation is possible at **3l** (upper panel), but not at **2l** (lower panel).

material, reagents and solvents were purchased from Aldrich Chemical Co.

*5.1.1. General procedure for synthesis of new compounds **1l, 2l–2r, 3l, 4l, 5l***

The appropriate 2-amino-benzo[*d*]isothiazol-3-one **1** and **4** or the acid hydrazide **2**, **3** and **5** (5 mmol) was poured into water (70 ml), under stirring, and hydrochloric acid was added up to acidic pH; the suspension was then buffered with sodium acetate, and ethanol (20 ml) was added. The suitable aldehyde (5.7 mmol), dissolved in ethanol (10 ml), was dropped into the mixture and the reaction was left, while stirring, at room temperature (60 min) for compounds **2m**, **2n**, **2o**, **2p**, **2q** or heated at 70°C for 3 h and then cooled at room temperature for compounds **1l**, **2l**, **2r**, **3l, 4l**, **5l**. The reaction was followed by TLC using as eluents: ethyl acetate:hexane1:1 (**2m**, **2n**, **2o**, **2p**, **2q**), CH_2Cl_2:EtOH 98:2 (**1l**, **2l**, **4l**, **5l**) or CH_2Cl_2:EtOH 9:1 (**2r**, **3l**). The resulting crude product was filtered, washed with water and recrystallised from ethanol (or 1,4-dioxane if expressly specified).

5.2. Biology

Samples were solubilized in DMSO at 100 mM and then diluted into culture medium.

5.2.1. Cells

Cell lines were purchased from American Type Culture Collection (ATCC). Haematological tumour-derived cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin. Solid tumour-derived cells were grown in their specific media supplemented with 10% FCS and antibiotics. Cell cultures were incubated at 37 °C in a humidified, 5% CO_2 atmosphere. The absence of mycoplasma contamination was checked periodically by the Hoechst staining method.

5.2.2. Virus

Human immunodeficiency virus type 1 (HIV-1) was obtained from supernatants of persistently infected $H9/III_B$ cells. The HIV-1 stock solution had a titre of 1.0×10^7 50% cell culture infectious dose ($CCID_{50}$)/ml.

5.2.3. Cytotoxicity assays

For cytotoxicity evaluations, exponentially growing cells derived from human haematological tumours [$CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4); $CD4^+$ human acute T-lymphoblastic leukaemia (CCRF-CEM), Human splenic B-lymphoblastoid cells (WIL-2NS), Human acute B-lymphoblastic leukaemia (CCRF-SB)] were seeded at an initial density of 1×10^5 cells/ml in 96 well plates in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin. Human cell lines derived from solid tumours [skin melanoma (SK-28), breast adenocarcinoma (MCF-7), lung squamous carcinoma (SK-MES-1), hepatocellular carcinoma (HepG-2), prostate carcinoma (DU-145)] or normal tissues [foreskin fibroblasts (CRL-7065), lung fibroblasts (CCL.75) and (MRC-5)] were also seeded at 1×10^5 cells/ml in 96 well plates in specific media supplemented with 10% FCS and antibiotics as above. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 h at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [14].

5.2.4. Antiviral assay

Activity of the compounds against Human Immunodeficiency virus type-1 (HIV-1) was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infections (m.o.i.) of 0.01. Briefly, 50 μl of RPMI containing 1×10^4 MT-4 were added to each well of flat-bottom microtitre trays containing 50 μl of RPMI, with or without serial dilutions of test compounds. Then, 20 μl of an HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4-day incubation, cell viability was determined by the MTT method.

5.3. Linear regression analysis

Viral and cell growth at each drug concentration was expressed as percentage of untreated controls, and the concentrations resulting in 50% (IC_{50}) growth inhibition were determined by linear regression analysis.

5.4. Conformational analysis

Conformational analysis was carried out using Chem-X 2000.1 [15]. Torsions N8C7C10C27 and C7C10C27O25 were subject to systematic scan in 12 steps. VdW energy was calculated for each conformation, and only conformations which lay within 20 kcal of the global energy minimum were considered. The local minima underwent further MM optimisation using

MM2 force field. Results are given in 2D maps; both torsions are plotted on axes. The energy levels are colour-coded.

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