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Original article

# Synthesis and biological activity of alpha-bromoacryloyl lexitropsin conjugates

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**Abstract**

The design, synthesis and biological evaluation of lexitropsins bearing mixed heterocyclic and benzoheterocyclic moieties and tethered to an alpha-bromo acrylic moiety acting as alkylating moiety are reported, and structure–activity relationships determined. With respect to antiproliferative activity against L1210 and K562 cells, compounds **7** and **10** showed the greatest potency, while compounds **4** and **5** exhibit the lowest activity. Among the synthesized compounds **4**–**12**, the derivative **10** was found to be the most potent member of this class and it is 70-fold more active than the bis-pyrrole counterpart **3** against L1210 cell line. In addition, the cytotoxicity of derivatives **5**–**12** against KB cells and the influence of different glutathione (GSH) concentrations on the cytotoxic effects was also investigated.


*Keywords:* Glutathione; benzoheterocycle; Alpha-bromoacryloyl; DNA minor groove binders

## 1. Introduction

Small molecular weight agents with alkylating properties represent a promising class of potent antitumor agents. Among them, DNA minor groove binders constitute a relatively new class of anticancer agents, reported to exhibit high cytotoxic activity in in vitro and in vivo preclinical model systems [1,2]. This group includes distamycin A **1**, a naturally occurring oligopeptidic antibiotic containing a pyrrolocarbamoyl frame terminating with an amidino moiety [3]. Distamycin A has been used as vector of alkylating functions, such as nitrogen mustard, aziridine, oxirane, halogen acetyl and α-halogen acrylic functions, leading to a substantial increase of cytotoxicity in comparison to that of distamycin itself [4]. When tested against L1210 leukemia cell line, the α-bromoacryloyl derivative of desformyldistamycin A **2** [5] showed very good antileukemic activity, being 125-fold more potent than distamycin A alone (showing $IC_{50}$ values of 80 and 10.02 nM, respectively), while the corresponding two-pyrrole analogue **3** [6] showed a 16-fold reduced cytotoxicity than **2** ($IC_{50}$ = 1300 and 80 nM, respectively). Compounds **2** and **3** are characterized by an alkylating moiety unusual for cytotoxics, in fact α-bromoacrylic acid is not per se cytotoxic ($IC_{50}$ on L1210 cells being superior to120 μM). Derivatives **2** and **3** were unable to alkylate any DNA sequence in different in vitro assays, suggesting that α-bromoacryloyl distamycin A derivatives are a new class of minor groove binders acting through mechanism different from a direct DNA alkylation [7,8]. The reactivity of the α-bromoacrylic moiety has been hypothesized to be based on a first-step Michael-type nucleophilic attach, followed by further substitution reaction of the no more vinylic halogen [9]. Since polypyrrole compounds may be susceptible to oxidative breakdown, the replacement of one or both pyrrolic rings with other heterocycles or benzohererocyclics, could generate potentially more stable compounds [10–12], with an improved biovability and pharmacodynamic profile [13].

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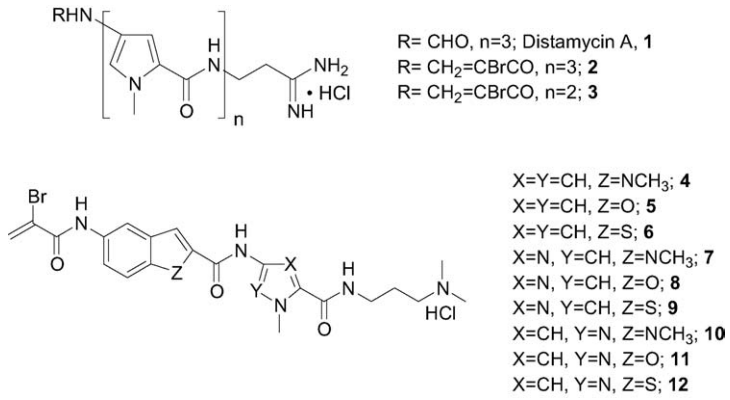
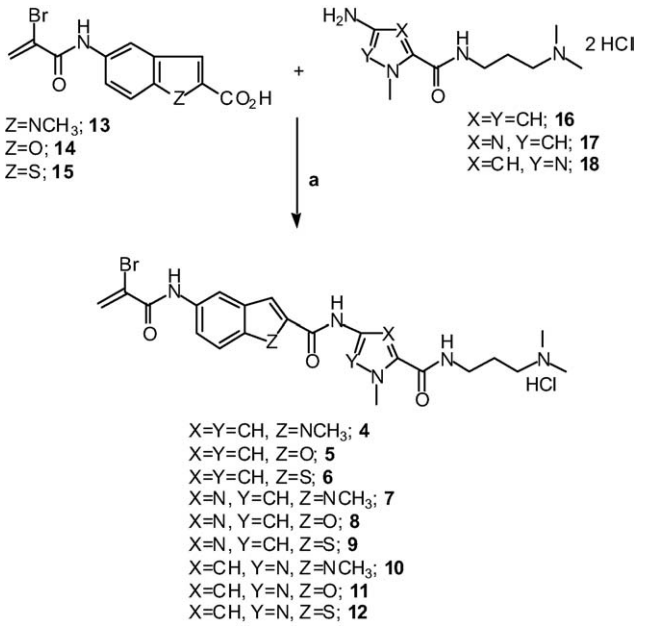



Fig. 1. Chemical structures compounds **1**–**12**.

Following these results, we report in the present study the synthesis and the biological evaluation of a novel series of analogs **4**–**12** (Fig. 1) of the compound **2**, in which the C-terminal pyrrole residue was replaced by isosteric pyrazole or imidazole rings which incorporate a dimethylamino propyl group, while the pyrrole unit directly acylated by the α-bromoacryloyl alkylating unit was substituted by different benzoetherocycles, such as *N*-methyl indole, benzofurane or benzothiophene (Fig. 1).

## 2. Chemistry

The synthetic route followed for the synthesis of derivatives **4**–**12** is outlined in Scheme 1. The key step was the coupling between the benzoheterocyclic carboxylic acids **13**–**15**, bearing the α-bromoacryloyl moiety, and the appropriate amino-heterocyclic moieties **16**–**18**. The condensation of the acylating agents **13**–**15** with amines **16**–**18** was performed using an excess (2 equiv.) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCl) as coupling agent, in dry DMF as solvent, in the presence of Hunig's base, at room temperature and with identical reaction times (18 h). Compounds **4**–**12** were prepared in acceptable yields, after purification by silica gel flash-chromatography.




Scheme 1. Reagents. a. EDCI, Hunig's base (DIPEA), DMF.

## 3. Pharmacology

All synthesized compounds **4**–**12**, along with Distamycin A **1**, were evaluated in vitro for their inhibitory effects on proliferation of both mouse L1210 and human K562 leukemia cells, while the α-bromoacryloylamide distamycin derivative **2** and its homologue **3** were only tested on L1210 cells. The results, expressed as $IC_{50}$ values (the concentration of tested compounds leading to 50% inhibition of cell growth), are reported in Table 1.

## 4. Results and discussion

The derivatives **4**–**12** showed an activity ranging between 20 and 80 nM against murine L1210 leukemia cell line, while for the human K562 leukemia cell line the $IC_{50}$ values ranged

Table 1
In vitro antiproliferative activities against murine L1210 and human K562 leukemia cell lines

| Compound | $IC_{50}$ (nM) | |
|---|---|---|
| | L1210 | K562 |
| **1** | 10.016 ± 1100 | 18.250 ± 2200 |
| **2** | 79.6 ± 22.6 | n.d. |
| **3** | 1300 ± 76 | n.d. |
| **4** | 76.5 ± 24.2 | 493 ± 11.6 |
| **5** | 79.8 ± 34.8 | 436 ± 91.5 |
| **6** | 44.4 ± 25.7 | 306 ± 27.4 |
| **7** | 25.5 ± 8.2 | 78.7 ± 13.7 |
| **8** | 66.4 ± 12.8 | 263 ± 32.1 |
| **9** | 41.7 ± 11.5 | 196 ± 7.07 |
| **10** | 18.5 ± 5.4 | 55.2 ± 20.1 |
| **11** | 42.3 ± 13.1 | 133 ± 11.5 |
| **12** | 40.1 ± 10.1 | 227 ± 49.9 |

$IC_{50}$ = 50% inhibitory concentration represents the mean ± S.D. from dose–response curves of at least three experiments. n.d = not determined.

Table 2
Cytotoxic activity of compounds **5**–**12** against KB cells alone and with GSH

| Compound | Compound Alone (μM) | Compound + 1:1 GSH (μM) | Compound + 1:10 GSH (μM) |
|---|---|---|---|
| **1** | > 20 | Not tested | Not tested |
| **5** | 0.23 ± 0.09 | 0.27 ± 0.11 | 3.43 ± 1.08 |
| **6** | 0.63 ± 0.16 | 1.14 ± 0.16 | Not active [a] |
| **7** | 0.088 ± 0.035 | 0.23 ± 0.7 | 1.94 ± 0.88 |
| **8** | 0.72 ± 0.34 | 0.74 ± 0.11 | 2.36 ± 0.90 |
| **9** | 1.25 ± 0.25 | 2.46 ± 0.61 | Not active [a] |
| **10** | 0.18 ± 0.03 | 0.21 ± 0.7 | 1.66 ± 0.40 |
| **11** | 0.25 ± 0.11 | 0.25 ± 0.13 | 0.90 ± 0.18 |
| **12** | 1.35 ± 0.23 | 3.21 ± 0.53 | Not active [a] |

[a] = not active up to 10 μg/ml.

between 80 and 500 nM. These results indicated that all these derivatives were more active against murine with respect to the human leukemic cell line.

When tested on L1210 cells, all the synthesized compounds **4**–**12** resulted from 16- to 72-fold more active than bis-pyrrole counterpart **3**, while several agents showed activity higher than or comparable with that of the α-bromoacryloylamide distamycin derivative **2**. The greatest potency and broadest spectrum of activity against both human and murine leukemia cell lines were exhibited by the derivatives **7** and **10**, ranging from 18 to 80 nM.

The replacement of the pyrrolic ring joined to the α-bromoacryloyl moiety of the reference compound **3** by benzoheterocycles, such as *N*-methyl indole, benzofurane or benzothiophene, to generate derivatives **4**–**6**, respectively, led to an improvement in terms of cytotoxic activity. When tested on L1210 cells, compounds **4** and **5** resulted 16-fold more active than **3**, while derivative **6** was 30-fold more potent. Moreover, the role of heteroatom in the benzoheterocycle moiety on the antiproliferative effect is remarkable when the C-terminus pyrrolic ring was replaced with pyrazole or imidazole.

The most active compound in the series of derivatives **4**–**12** was compound **10**, which showed threefold higher potency on L1210 with respect to K562 cell line ($IC_{50}$ = 18.5 and 55.2 nM, respectively). In this compound, the replacement of the pyrazolic ring with an imidazole (compound **7**) had a slight effect on the antiproliferative effect against both on L1210 and K562 cell lines ($IC_{50}$ = 25.5 and 78.7 nM, respectively). The in vitro antiproliferative activity markedly decreased by the substitution of the pyrazole ring with a pyrrole, to yield the derivative **4**. This latter compound was four and ninefold less active, on both L1210 and K562 cell lines, than the pyrazolic counterpart **10**, respectively. The replacement of the *N*-methyl indole in the derivative **4** with a benzofuran (compound **5**), had no effect on the activity against both cell lines. As for derivative **10**, the replacement of the *N*-methyl indole tethered to the alkylating moiety with a benzofuran (compound **11**) halved the potency on both cell lines. This effect was not observed for the benzothiophene counterpart (compound **12**), which exhibited the same potency of **11** on L1210 cell line, but was twofold less active than **11** on K562 cells.

Starting from compound **12**, the replacement of the pyrazole with an imidazole, to give the isosteric derivative **9**, had no effects on the antiproliferative activity, which resulted substantially unmodified. This was also observed on L1210 cells, when the imidazole ring on compound **9** was substituted with a pyrrole (generating compound **6**). Nevertheless, the derivative **6** resulted 1.5-fold less active than **9** on K-562 cells. In this latter compound, the replacement of benzothiophene with a benzofuran (compound **8**) reduced the antiproliferative potency, which resulted 1.5-fold lower against both cell lines.

We have previously explained the role played by GSH against similar compounds to **5**–**12** [14]. For this reason, we investigated the influence of different GSH levels on the cytotoxicity of compounds **5**–**12** against the KB cell line (HeLa cells, ATCC CCL17). Initially, the $IC_{50}$ for cytotoxicity of all tested compounds against the KB cells was determined. Successively, the influence of two different GSH concentrations, added in molar ratios 1:1 and 10:1, on the cytotoxicity was calculated using the same cell line. An "anti-cytotoxic" effect of GSH was especially observed which is most likely due to the reaction with the α-bromoacryloyl moiety of the tested compounds **5**–**12**. The results reported in Table 2 show that the influence of GSH addition on the cytotoxicity depends on the concentration of GSH added. Especially the cytotoxicity of compound **7** is sensitive to the addition of GSH, resulting in two and 20-fold reduced activity at the 1:1 and 10:1 ratio, respectively (Table 2).

For the compounds **5**, **8**, **10** and **11**, the effects under the influence of equimolar amounts of glutathione are not very different from the effects without GSH, while the others derivatives are sensitive to the addition of GSH, showing a twofold reduced activity at the 1:1 ratio. In contrast, the cytotoxicity decreased remarkably under the influence of higher GSH concentrations (10.1 ratio). These results strongly suggest that in this range of concentration, the majority of the molecules are present in the form of a bis-adduct (see Fig. 2), that does not possess any reactive centers capable of causing deleterious effects to cellular structures. It is also interesting to note that the activity of the tested compounds is so differentially influenced by GSH.

## 5. Conclusion

In conclusion, the replacement of pyrrole joined to the α-bromoacryloyl moiety by benzoheterocycles lead to an

improvement in terms of cytotoxic activity. For the same benzoheterocycle joined to the alkylating unit, a correlation has been found between the kind of heterocycle and cytotoxic potency. An exception was represented by benzothiophene derivatives **6**, **9** and **12**, in which the antiproliferative activity on L1210 and K562 cell lines was not influenced by the heterocycle. In fact, the replacement of pyrrole ring with a pyrazole or imidazole appears devoid of substantial effects. For the others two group of compounds, corresponding to *N*-methyl indole (compounds **1**, **4** and **10**) and benzofuran (compounds **5**, **8** and **11**) derivatives, the activity decreased in the following order: pyrazole > imidazole >> pyrrole. One of the most active compounds on KB cells (derivative **11**) resulted cytotoxic ($IC_{50}$ = 0.9 μM on KB cells) also in presence of high concentration of GSH (molar ratio 10:1).

## 6. Experimental protocols

### *6.1. Chemistry*

All reactions were carried out under Argon atmosphere, unless otherwise described. Standard syringe techniques were applied for transferring anhydrous solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated $F_{254}$ Merck plates) and visualized with aqueous $KMnO_4$. Infrared spectra were recorded on a Perkin-Elmer 1710 spectrophotometer. $^1$HNMR spectra were obtained in DMSO solutions with a Bruker AC 200 spectrometer. Chemical shifts ($\delta$) are given in parts per million (ppm) upfield from tetramethylsilane. All products reported showed $^1$H-NMR spectra in agreement with the assigned structures. Matrix-assisted laser desorbion ionization time-of-flight (MALDI-TOF) mass spectrometry of all synthesized compounds was conducted using a Hewlett Packard G 2025A LD-TOF instrument. The samples were analyzed in the linear mode with 28 kV accelerating voltage, mixing them with a saturated solution of α-cyano-4-hydroxycinnamic acid matrix. Melting points (m.p.) were determined on a Buechi-Tottoli apparatus and are uncorrected. Elemental analyses were conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara. Analyses indicated by the symbols of the elements or functions were within ± 0.4% of the theoretical values. Column chromatography was carried out using Merck silica gel (230–240 mesh). All compounds obtained commercially were used without further purification. Organic solutions were dried over anhydrous $Na_2SO_4$. Anhydrous DMF was distilled from calcium chloride and stored over molecular sieves (3 Å).

#### *6.1.1. General procedure for the synthesis of compounds (**4**–**12**)*

A solution of amine **16**–**18** (0.5 mmol) in dry DMF (5 ml) was cooled to 5 °C and *N*, *N*′-diisopropylethylamine (86 μl, 0.5 mmol) was added. After 10 min, the acids **13**–**15** (0.6 mmol, 1.2 equiv.) and then EDC (192 mg, 1 mmol) were added. After 0.5 h at 0 °C, the reaction mixture was allowed to rise at room temperature and stirred for 18 h, then 1 N hydrochloric acid was added until pH 4. The solvent was evaporated in vacuo and the crude residue purified by flash chromatography to yield a solid, which was recrystallized with a mixture methanol/ethyl ether 1:30 (v/v).

#### *6.1.2. 3- [1-Methyl-4[1-methyl-5(α-bromoacrylamido)-indole-2-carboxamido]pyrrole-2-carboxamido] dimethylaminopropylamino hydrochloride (**4**)*

Following the general procedure, the compound **4** was prepared from 1-methyl-5-(α-bromoacrylamido)indole-2-carboxylic acid **13** [11] and 3-(*N*-methyl-4-aminopyrrole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **16** [15]. After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **4** was obtained as a yellow cream solid (63% yield); m.p. 218–220 °C. $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.86 (m, 2H), 2.51 (s, 6H), 3.06 (m, 2H), 3.33 (m, 2H), 3.84 (s, 3H), 4.01 (s, 3H), 6.29 (d, $J$ = 3.0 Hz, 1H), 6.76 (d, $J$ = 3.0 Hz, 1H), 6.98 (s, 1H), 7.20 (s, 2H), 7.51 (m, 2H), 8.05 (s, 1H), 8.24 (t, $J$ = 5.6 Hz, 1H), 10.1 (bs, 1H), 10.2 (s, 1H), 10.4 (s, 1H); FAB-MS (MALDI-TOF): 530.3 $[M]^+$. Anal $C_{24}H_{30}BrClN_6O_3$ (C, H, Br, Cl, N).

#### *6.1.3. 3- [4[1-Methyl-5(α-bromoacrylamido)benzofurane-2-carboxamido]pyrrole-2-carboxamido] dimethylaminopropylamino hydrochloride (**5**)*

Following the general procedure, the compound **5** was prepared from 5-(α-bromoacrylamido)benzofurane-2-carboxylic acid **14** [11] and 3-(*N*-methyl-4-aminopyrrole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **16**. [15] After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **5** was obtained as a yellow solid (67% yield); m.p. 193–195 °C. $^1$H-NMR(DMSO-$d_6$) $\delta$ 1.88 (m, 2H), 2.77 (s, 6H), 3.06 (m, 2H), 3.25 (m, 2H), 3.84 (s, 3H), 6.33 (d, $J$ = 2.8 Hz, 1H), 6.80 (d, $J$ = 2.8 Hz, 1H), 7.00 (s, 1H), 7.30 (s, 1H), 7.69 (m, 3H), 8.17 (s, 1H), 8.27 (t, $J$ = 5.4 Hz, 1H), 9.98 (bs, 1H), 10.4 (s, 1H), 10.7 (s, 1H); FAB-MS (MALDI-TOF): 533.5 $[M]^+$. Anal. $C_{23}H_{27}BrClN_5O_4$ (C, H, Br, Cl, N).

#### *6.1.4. 3- [4[1-Methyl-5(α-bromoacrylamido)-benzothiophene-2-carboxamido]pyrrole-2-carboxamido] dimethylaminopropylamino hydrochloride (**6**)*

Following the general procedure, the compound **6** was prepared from 5-(α-bromoacrylamido)benzothiophene-2-carboxylic acid **15** [16] and 3-(*N*-methyl-4-aminopyrrole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **16**. [15] After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **5** was obtained as a brown solid (71% yield); m.p. 112–114 °C. $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.85 (m, 2H), 2.77 (s, 6H), 3.06 (m, 2H), 3.24 (m, 2H), 3.84 (s, 3H), 6.35 (d, $J$ = 2.8 Hz, 1H), 6.80 (d, $J$ = 2.8 Hz, 1H), 6.96 (s, 1H), 7.28 (s, 1H), 7.68 (dd, $J$ = 8.8 and 2.2 Hz, 1H), 7.98 (d,

*J* = 8.8 Hz, 1H), 8.21 (s, 1H), 8.26 (t, *J* = 5.4 Hz, 1H), 8.36 (s, 1H), 9.67 (bs, 1H), 10.5 (s, 1H), 10.6 (s, 1H); FAB-MS (MALDI-TOF): 517.5 $[M]^+$. Anal. $C_{23}H_{27}BrClN_5O_3S$ (C, H, Br, Cl, N).

*6.1.5. 3- [1-Methyl-4[1-methyl-5(α-bromoacrylamido)indole-2-carboxamido]imidazole-2-carboxamido] dimethylaminopropylamino hydrochloride (**7**)*

Following the general procedure, the compound **7** was prepared from 1-methyl-5-(α-bromoacrylamido)indole-2-carboxylic acid **13** [11] and 3-(*N*-methyl-4-aminoimidazole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **17** [10]. After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **7** was obtained as a yellow cream solid (55% yield); m.p. 137–138 °C. $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.92 (m, 2H), 2.51 (s, 6H), 3.05 (m, 2H), 3.32 (m, 2H), 3.96 (s, 3H), 4.01 (s, 3H), 6.29 (d, *J* = 2.8 Hz, 1H), 6.76 (d, *J* = 2.8 Hz, 1H), 7.35 (s, 1H), 7.46 (d, *J* = 9.2 Hz, 1H), 7.53 (d, *J* = 9.2 Hz, 1H), 7.62 (s, 1H), 8.06 (s, 1H), 8.22 (t, *J* = 5.6 Hz, 1H), 10.3 (s, 1H), 10.4 (bs, 1H), 10.8 (s, 1H); FAB-MS (MALDI-TOF): 532.1 $[M + 1]^+$. Anal. $C_{23}H_{29}BrClN_7O_3$ (C, H, Br, Cl, N).

*6.1.6. 3-[4[1-Methyl-5(α-bromoacrylamido)benzofurane-2-carboxamido]imidazole-2-carboxamido] dimethylaminopropylamino hydrochloride (**8**)*

Following the general procedure, the compound **8** was prepared from 5-(α-bromoacrylamido)benzofurane-2-carboxylic acid **14** [11] and 3-(*N*-methyl-4-aminoimidazole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **17** [17]. After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **8** was obtained as a brown solid (72% yield); m.p. 184–186 °C. $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.97 (m, 2H), 2.69 (s, 6H), 3.02 (m, 2H), 3.26 (m, 2H), 3.74 (s, 3H), 6.33 (d, *J* = 2.8 Hz, 1H), 6.80 (d, *J* = 2.8 Hz, 1H), 7.53 (s, 1H), 7.64 (m, 3H), 8.15 (s, 1H), 8.40 (t, *J* = 5.4 Hz, 1H), 10.0 (bs, 1H), 10.4 (s, 1H), 10.6 (s, 1H); FAB-MS (MALDI-TOF): 518.5 $[M]^+$. Anal. $C_{22}H_{26}BrClN_6O_4$ (C, H, Br, Cl, N).

*6.1.7. 3-[4[1-Methyl-5(α-bromoacrylamido)benzothiophene-2-carboxamido]imidazole-2-carboxamido] dimethylaminopropylamino hydrochloride (**9**)*

Following the general procedure, the compound **9** was prepared from 5-(α-bromoacrylamido)benzothiophene-2-carboxylic acid **15** [16] and 3-(*N*-methyl-4-aminoimidazole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **17**. [17] After work-up, the residue was purified by flash chromatography using methanol: methylene chloride 2:8 (v:v) as eluent. The compound **9** was obtained as a white solid (74% yield); m.p. 193–195 °C. $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.87 (m, 2H), 2.74 (s, 6H), 3.02 (m, 2H), 3.26 (m, 2H), 3.88 (s, 3H), 6.33 (d, *J* = 3.2 Hz, 1H), 6.80 (d, *J* = 3.2 Hz, 1H), 7.63 (s, 1H), 7.68 (d, *J* = 8.6 Hz, 1H), 7.98 (d, *J* = 8.6 Hz, 1H), 8.37 (s, 1H), 8.46 (s, 1H), 8.52 (t, *J* = 5.4 Hz, 1H), 9.93 (bs, 1H), 10.4 (s, 1H), 10.5 (s, 1H); FAB-MS (MALDI-TOF): 534.3 $[M]^+$. Anal. $C_{22}H_{26}BrClN_6O_3S$ (C, H, Br, Cl, N).

*6.1.8. 3-[1-Methyl-3[1-methyl-5(α-bromoacrylamido)indole-2-carboxamido]pyrazole-5-carboxamido] dimethylaminopropylamino hydrochloride (**10**)*

Following the general procedure, the compound **10** was prepared from 1-methyl-5-(α-bromoacrylamido)indole-2-carboxylic acid **13** [11] and 3-(*N*-methyl-3-aminopyrazole-5-carboxamido)-dimethylaminopropylamino dihydrochloride **18** [10]. After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **10** was obtained as a brown solid (52% yield); m.p. 135–136 °C $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.90 (m, 2H), 2.76 (s, 6H), 3.06 (m, 2H), 3.32 (m, 2H), 4.01 (s, 3H), 4.03 (s, 3H), 6.29 (d, *J* = 2.8 Hz, 1H), 6.77 (d, *J* = 2.8 Hz, 1H), 7.34 (s, 1H), 7.42 (s, 1H), 7.50 (d, *J* = 9.0 Hz, 1H), 7.54 (d, *J* = 9.0 Hz, 1H), 8.03 (s, 1H), 8.73 (t, *J* = 5.6 Hz, 1H), 10.0 (bs, 1H), 10.2 (s, 1H), 11.0 (s, 1H); FAB-MS (MALDI-TOF): 532.5 $[M + 1]^+$. Anal. $C_{23}H_{29}BrClN_7O_3$ (C, H, Br, Cl, N).

*6.1.9. 3-[3[1-Methyl-5(α-bromoacrylamido)benzofurane-2-carboxamido]pyrazole-5-carboxamido] dimethylaminopropylamino hydrochloride (**11**)*

Following the general procedure, the compound **11** was prepared from 5-(α-bromoacrylamido)benzofurane-2-carboxylic acid **14** [11] and 3-(*N*-methyl-3-aminopyrazole-5-carboxamido)-dimethylaminopropylamino dihydrochloride **18** [10]. After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **11** was obtained as a white solid (68% yield); m.p. 103–105 °C $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.89 (m, 2H), 2.74 (s, 6H), 3.07 (m, 2H), 3.31 (m, 2H), 4.03 (s, 3H), 6.33 (d, *J* = 3.0 Hz, 1H), 6.80 (d, *J* = 3.0 Hz, 1H), 7.28 (s, 1H), 7.68 (m, 2H), 7.90 (s, 1H), 8.17 (s, 1H), 8.77 (t, *J* = 5.4 Hz, 1H), 10.1 (bs, 1H), 10.4 (s, 1H), 11.2 (s, 1H); FAB-MS (MALDI-TOF): 518.4 $[M]^+$. Anal. $C_{23}H_{29}BrClN_7O_3$ (C, H, Br, Cl, N).

*6.1.10. 3-[3[1-Methyl-5(α-bromoacrylamido)-benzothiophene-2-carboxamido]pyrazole-5-carboxamido] dimethylaminopropylamino hydrochloride (**12**)*

Following the general procedure, the compound **12** was prepared from 5-(α-bromoacrylamido)benzothiophene-2-carboxylic acid **15** [16] and 3-(*N*-methyl-4-aminopyrazole-2-carboxamido)-dimethylaminopropylamino dihydrochloride **18**. [10] After work-up, the residue was purified by flash chromatography using methanol/methylene chloride 2:8 (v:v) as eluent. The compound **12** was obtained as a white solid (71% yield); m.p. 143–144 °C $^1$H-NMR (DMSO-$d_6$) $\delta$ 1.88 (m, 2H), 2.50 (s, 6H), 2.96 (m, 2H), 3.24 (m, 2H), 3.88 (s, 3H), 6.33 (d, *J* = 3.0 Hz, 1H), 6.79 (d, *J* = 3.0 Hz, 1H), 7.64 (s, 1H), 7.69 (d, *J* = 8.6 Hz, 1H), 7.98 (d, *J* = 8.6 Hz, 1H),

8.32 (s, 1H), 8.52 (s, 1H), 8.82 (t, $J$ = 5.4 Hz, 1H), 10.0 (bs, 1H), 10.2 (s, 1H), 10.4 (s, 1H); FAB-MS (MALDI-TOF): 534.5 $[M]^+$. Anal. $C_{22}H_{26}BrClN_6O_3S$ (C, H, Br, Cl, N).

## 7. Biological evaluation

### *7.1. Growth inhibitory activity on murine L1210 and human K562 cells*

The murine lymphocytic L1210 leukemia [18,19] and the human chronic myelogenous K562 [20] cell lines were obtained from the American Type Culture Collection (ATCC). All the tested compounds were dissolved in DMSO at 1 mg/ml immediately before the use and diluted in medium before addition to the cells. Both cell lines were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FCS (Flow, Irvine, UK), 2 mM L-glutamine (GIBCO), 10 mM β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. To determine the effects of the studied compounds on in vitro cell growth, exponentially growing L1210 and K562 cells were exposed to increasing concentrations of drugs and the value of cell number per ml was determined after 48 h of cell culture using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Results were expressed as $IC_{50}$ (dose causing 50% inhibition of cell growth in treated cultures relative to untreated controls) [18,19]. All experiments were repeated at least three times. For each drug concentration, duplicate cultures were used.

### *7.2. Cytotoxicity study using KB cells*

KB cells (ATCC CCL 17; HeLa cells) were cultured in minimum essential medium (Gibco, Life Technologies, Switzerland) supplemented with 10% fetal bovine serum (FBS), 1 μg/ml fungizone (amphotericin B), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5 ml of a non essential amino acids solution (all from Gibco, Life Technologies) at 37 °C and 5% $CO_2$ in 50 ml culture flasks (TPP). The test was carried out with some modifications according to the screening technique of Swanson and Pezzuto [21] in 96-well plates (Falcon) with an inoculum of $5 \times 10^3$ cells per well. Test solutions were made as stocks in ethanol. Test concentrations were freshly prepared by diluting the stock solution with water to the required concentration. Final ethanol concentration was 1% (v/v) or less. Total assay volume was 150 μl. Test compounds and respective GSH concentrations were added simultaneously. For quantification of the cytotoxicity, 15 μl of an aqueous solution of methylthiazolyltetrazolium chloride (MTT, Fluka, 5 mg/ml in PBS) was added after 72 h. After incubation at 37 °C for 4 h the culture medium was drawn off and the formazan dye was dissolved using 150 μl of 10% SDS (sodium dodecylsulfate) in water. After 24 h of incubation at room temperature, the optical density was measured at 540 nm using a microplate reader (MRX, Dynex Technologies). For determination of the $IC_{50}$ values, the optical density was plotted against the log concentration and six different concentrations have been tested. Every test was performed at least in duplicates and all experiments have been repeated at least three times. Maximal observed standard deviation was about 15% (absolute) [22].

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