α-Halogenoacrylic Derivatives of Antitumor Agents

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Abstract. In this review article we have reported a series of hybrid compounds characterized by the presence of a α-halogenocryloyl alkylating moiety of low chemical reactivity, linked to known antitumor agents or their active moieties. Among them, brostallicin (PNU-166196), was selected for clinical development and is now undergoing Phase II studies in patients with advanced or metastatic soft tissue sarcoma.

Key Words: Antitumor agents, Distamycin A, apoptosis, α-bromoacryloyl, hybrid molecules.

1. INTRODUCTION

Many natural and synthetic anticancer agents with the ability to interact with DNA have been discovered, but most of them display low sequence specificity and often exhibit severe toxicity to normal tissues [1]. However, cytotoxic agents will continue to represent in the future an essential part of the therapy against tumor cells. This consideration implies a need for novel cytotoxics, exhibiting greater or broader activity and lower toxicity.

The pyrroloiminoquinone cytotoxic alkaloids Discorhabdin A (**1**), B (**2**), C (**3**), isolated as the major pigments from the three sponge species of the genus *Lutrunculia* du Bogage (family Latruculiidae), are strongly cytotoxic *in vitro* with IC_{50} values against the P388 cell line in the range 0.03-0.01 μg/mL but in the *in vivo* P388 model were found to be inactive (T/C <120%) [2-4].

In continuation of the search for bioactive metabolites, by the examination of organic extracts of four new species of South African latrunculid sponges, a new series of pyrroloiminoquinone metabolites have been identified, corresponding to 14-bromodiscorhabdin C (**4**) and discorhabdin G (**5**), isolated from *T. pedunculata* and *L. apicalis*, respectively (Fig. (**1**)). Discorhabdin A, with an IC_{50} of 7 nM against HCT-116 human colon tumor cell line, resulted 10-fold more potent than 14-bromodiscorhabdin C (IC_{50}=77 nM) [5].

These compounds, representatives of a new class of alkaloids, are characterized by the presence of a α-bromoacryloyl alkylating moiety of low chemical reactivity and unusual for cytotoxic compounds. In fact the α-bromoacrylic acid was devoid of cytotoxic activity (IC_{50} on L1210 cells being superior to120 μM).

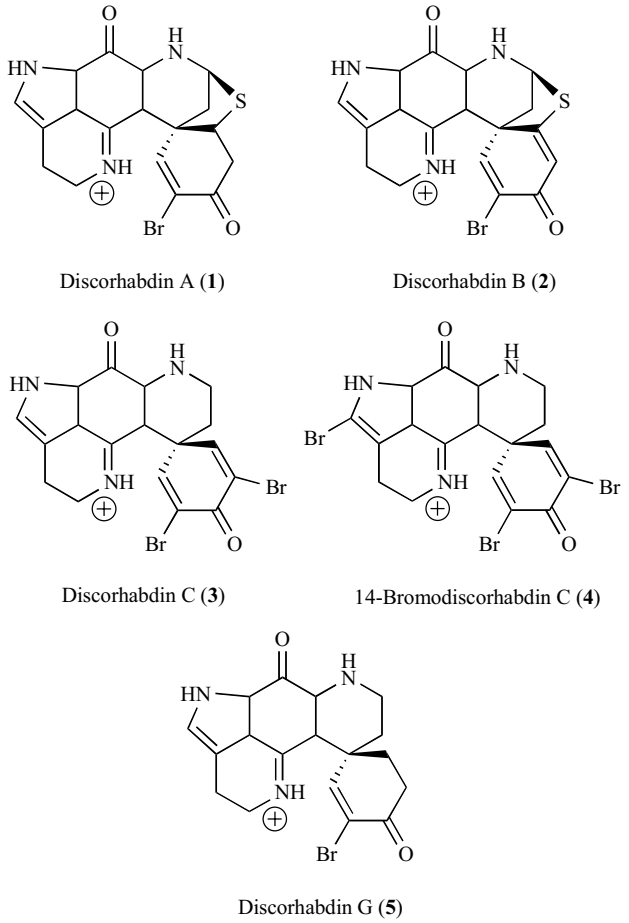

Fig (1). Structure of pyrroloiminoquinone cytotoxic alkaloids discorhabdin A (**1**), B (**2**), C(**3**), 14-bromodiscorhabdin C (**4**) and discorhabdin G (**5**).

2. α-BROMOACRYLOYL DISTAMYCIN A DERIVATIVES

The tripyrrole peptide distamycin A **6** [6] is a naturally occurring antibiotic agent isolated in 1962 from the cultures of *Streptomyces distallicus* [7] with interesting antibacterial and antiviral activities (but inactive as antitumor agent). More recently, distamycin A was found to possess antiprotozoal activity, in particular against sensitive and resistant strains of *Plasmodium falciparum* [8].

Distamycin A (Fig. (**2**)) is characterized by the presence of an oligopeptidic pyrrolecarbamoyl frame ending with an

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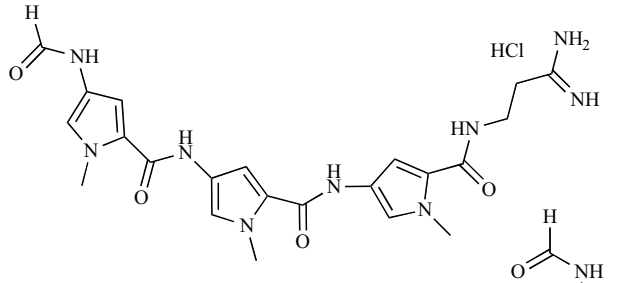
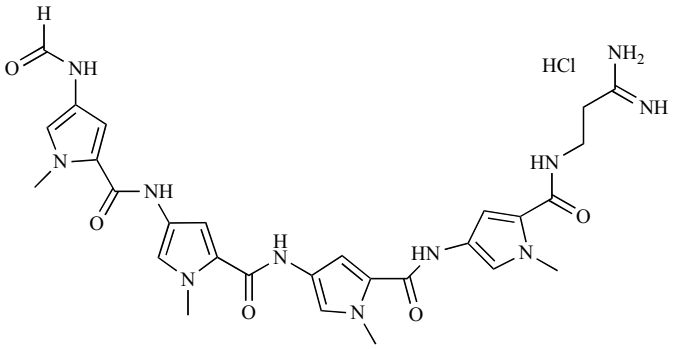
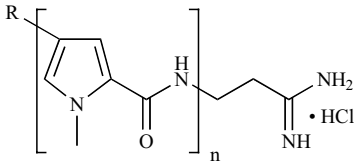


Distamycin A, **6**

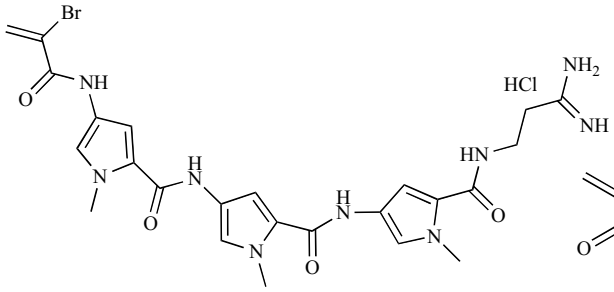


Distamycin A homologue, **7**



n=3 or 4
R= 4-[(ClCH$_2$CH$_2$)$_2$N]C$_6$H$_4$CONH, CH$_2$(O)CHCONH
4-[(ClCH$_2$CH$_2$)$_2$N]C$_6$H$_4$(CH$_2$)$_3$CONH, (ClCH$_2$CH$_2$)$_2$N,
CH$_2$=CXCONH with X=F, Cl or Br.
8a (Tallimustine): R= 4-[(ClCH$_2$CH$_2$)$_2$N]C$_6$H$_4$CONH .

8



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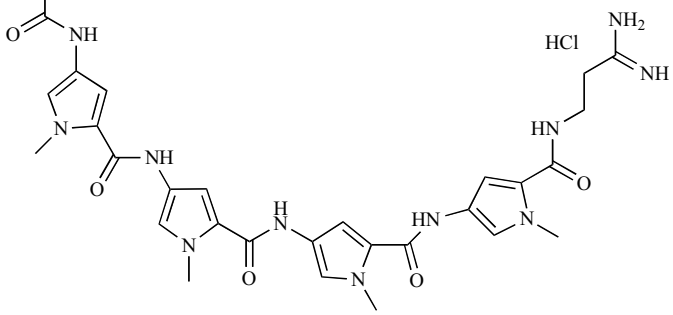


10, PNU-151807

Fig. (2). Structure of compounds **6-10**.

amidino moiety, which reversibly binds to the minor groove of DNA by hydrogen bonds, van der Waals contacts and electrostatic interactions with a strong preference for adenine-thymine (AT) rich sequences containing at least four AT base pairs [9]. The tetrapyrrole distamycin A homologue **7**, although exerting very low cytotoxic activity on L1210 leukaemia cell line, is almost 20-fold more active than the distamycin A and increasing the number of pyrrole units of the oligopeptidic frame increases the sequence specificity for longer tracts of AT-rich DNA.

With the objective to identify novel promising candidates, distamycin A **6** and its four pyrroles homologue **7** have been used as a DNA sequence selective carrier of different electrophilic moieties, in which the formyl group has been substituted by 4-[bis(2-chloroethyl)amino]benzoyl (benzoy l nitrogen mustard or BAM), nitrogen mustard, halogenoacryloyl and epoxycarbonyl moieties, leading to compounds with general formula **8**, which are substantially more cytotoxics than distamycin A and **7** themselves, respectively [10, 11].

While both BAM and nitrogen mustard distamycin A derivatives are characterized by the presence of classical alkylating moieties, the α-halogenoacrylic derivatives are constituted by a moiety unusual in medicinal chemistry, since α-bromoacrylic acid is not *per se* cytotoxic but able to interact with biological nucleophiles.

As previously reported for distamycin A and four pyrroles distamycin homologue **7**, comparing **9**, the α-bromoacryloyl derivative of desformyldistamycin, and its tetrapyrrole homologue **10** (PNU151807), increasing the number of pyrrole rings in each class of derivatives, an increase of the *in vitro* cytotoxicity and *in vivo* potency on L1210 murine leukaemia cells has been observed (Table **1**) [12]. The α-bromoacryloyl derivative of desformyldistamycin A **9** showed a potent antiproliferative activity against L1210 leukemia cell line, being 125-fold more potent than distamycin A alone (IC_{50} values of 80 and 10,02 nM, respectively).

Even if **10** interacts non covalently with AT-rich regions with the same profile previously described for tallimustine **8a** or distamycin, **10** was found unable to alkylate any DNA sequence in different *in vitro* assays, suggesting that α-bromoacryloyl derivatives are a new class of minor groove binders acting through mechanism different from a direct DNA alkylation [12]. The cell cycle perturbation induced by PNU 151807 in ovarian carcinoma A2780 cells, treated with the respective IC_{50} for 1 h, produced an accumulation of cells in the G2-phase.

Considering that tallimustine was characterized by clinical haematological toxicity [13, 14], from studies on haematologycal precursors maintained *in vitro*, PNU 151807 had a much lower toxicity and an improved therapeutic window than tallmustine [14].

α-Bromoacryloyl Heterocyclic and Benzoheterocyclic Derivatives of Distamycin A

Our group described the synthesis and the activity of two isosteric derivatives of PNU 151807, in which the N-methyl pyrrole directly linked to the α-bromoacryloyl moiety, was replaced by other pentaatomic heteroaromatic rings, mainly N-methyl pyrazole or imidazole, to furnish the derivatives **11** and **12**, respectively [15]. Both these isosteres showed an antiproliferative activity against L1210 murine leukemia cell line, which was lower to that exhibited by PNU 151807. In particular, the pyrazole derivative **11** appeared about two fold less potent than its pyrrole counterpart **10**, while the bioisosteric imidazole **12** resulted 6-fold less active than **10** (Table **2**).

Compounds **11** and **12** not only exhibited good activity in *in vitro* experiments, but also showed a significant increase in survival time (%T/C) of mice bearing lymphocytic leukemia model (L1210). Both these derivatives were 2-fold less potent than PNU 151807, (optimal non toxic dose (O.D), O.D.= 6.2 mg/Kg for **11** and **12** *vs*. O.D.=3.13 mg/Kg for **10**), with a median survival time considerably lower (%T/C= 200 for **11**, %T/C= 163 for **12** *vs*. %T/C=725 for PNU 151807). Moreover, **11** and **12** possessed a %T/C value comparable to that of tallimustine (%T/C= 175).

Following these results, a new series of compounds was synthesized, in which the pyrrole ring linked to the α-bromoacryloyl moiety of compound **10** was replaced by different benzoheterocycles [16]. Indole, N-methyl indole and benzofuran derivatives, corresponding to compounds **13**. **14** and **15**, respectively, showed comparable antiproliferative activities, while the benzothiophene derivative **16** resulted slightly less active (IC_{50}=4.1±1.3 nM, 2.4±0.4 nM, 6.1±0.4 nM and 14.3±5.9 nM for **13**, **14**, **15** and **16**, respectively).

In the same series **13-15**, although the indole derivative **13** showed the same cytotoxicity as the benzofuran counterpart **15**, the latter compound was 15-fold less potent *in vivo* and produced an increased survival time 2-fold higher than that of **13** (O.D.=0.78 mg/Kg, %T/C=117 for **15** *vs*. O.D.=12.5 mg/Kg, %T/C=213 for **13**). The same compounds **13-15** maintained cytotoxicity substantially equivalent to that

Table 1. ***In Vivo*** **and** ***In Vitro*** **Activity of Compounds 6-10 Against L1210 Murine Leukemia**

Compound	*In Vitro* L1210	*In Vivo* [a]	
	IC_{50} (nM±S.E.)	O.D. (mg/Kg)	%T/C
Distamycin A (6)	10,069±1647	200	113
7	400±12	n.d	n.d
8a	68.5±6.6	3.1	175
9	79.6±14	12.5	175
10 (PNU 151807)	6.3±1.34	3.1	725

IC_{50}= 50% inhibitory concentration as the mean ±SE from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

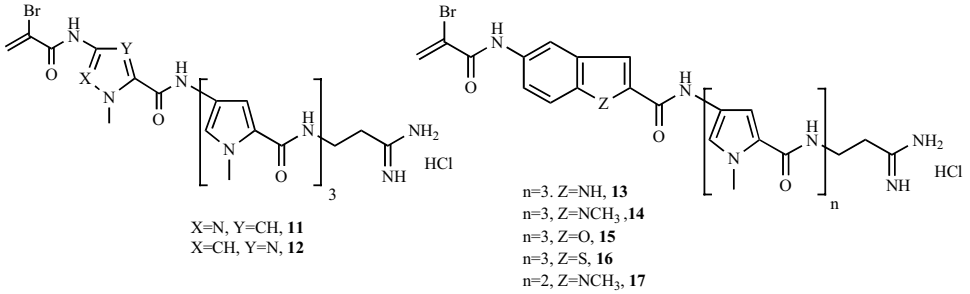
[a] For *in vivo* studies L1210 cells were injected iv at day 0 and mice were treated iv the day after tunor inoculum.

O.D= optimal dose; optimal non toxic dose<LD_{10} (weight loss <20% rispect to the starting weight)

%T/C= median survival time of treated vs. untreated mice x 100.

n.d.=not determined.

Table 2. ***In Vivo*** **and** ***In Vitro*** **Activity of 6, 8a and Distamycin Derivatives 9-17 Against L1210 Murine Leukemia**



X=N, Y=CH, **11**
X=CH, Y=N, **12**

n=3. Z=NH, **13**
n=3, Z=NCH_3 ,**14**
n=3, Z=O, **15**
n=3, Z=S, **16**
n=2, Z=NCH_3, **17**

Compound	*In Vitro* L1210	*In Vivo* L1210			
		i.p.		i.v.	
	IC_{50} (nM±S.E.)	O.D. (mg/Kg)	%T/C	O.D. (mg/Kg)	%T/C
Distamycin A (6)	10,069±1647	200	113	n.d	n.d
Tallimustine (8a)	68.5±6.6	3.1	175	3.1	133
9	79.6±14	12.5	175	n.d	n.d
10	6.3±1.34	3.1	725	1.6	200
11	13.3±0.54	6.2	200	n.d	n.d
12	46.9±13	6.2	163	n.d	n.d
13	4.1±1.3	n.d.	n.d.	12.5	213
14	2.4±0.39	n.d.	n.d.	6.2	192
15	6.1±0.93	n.d.	n.d.	0.78	117
16	14.3±5.89	n.d.	n.d.	n.d.	n.d.
17	15.3±4.31	n.d.	n.d.	3.1	133

IC_{50}= 50% inhibitory concentration as the mean ±SE from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

i.p.= treatment was performed intraperitoneally on day 1 after tumor i.p. transplant.

i.v.= treatment was performed intravenously on day 1 after tumor i.v. transplant.

O.D= optimal dose; optimal non toxic dose<LD_{10} (weight loss <20% rispect to the starting weight)

%T/C= median survival time of treated vs. untreated mice x 100.

n.d.=not determined.

of the pyrrole **10** and pyrazole **16** analogues, but much higher with respect to the imidazole derivative **12**.

A fairly marked correlation between antitumor activity and length of the polypyrrolic skeleton was observed for compounds **14** and **17**, where the derivative **17** with two N-methylpyrrolic units was 6-fold less cytotoxic than the corresponding pyrrole homologue **14**. Derivative **17**, in spite of a potency 2-fold higher than **14** (O.D. mg/Kg, 6.25 *vs*. 3.13), was slightly less effective *in vivo* (T/C%, 192 *vs*. 133 for **14** and **17**, respectively).

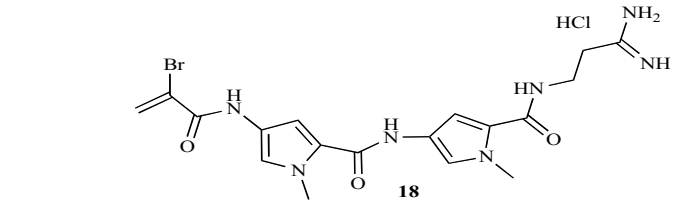
In a preliminary *in vivo* evaluation against the M5076 solid tumor, compound **14** showed an. O.D. of 3.13 mg/Kg, with an increased %T/C of 154 and an inhibition of tumour growth (%TI) of 96.

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 [17-19], with an improved bioavailability and pharmacodynamic profile [20].

Following these results, a series of molecules bearing mixed heterocyclic and benzoheterocyclic moieties and tethered to the α-bromoacrylic moiety was reported [17]. Specifically, different benzoetherocycles, such as *N*-methyl indole, benzofuran or benzothiophene were N-acylated by the α-bromoacryloyl moiety, while pyrazole or imidazole rings incorporated a dimethylamino propyl group.

Compounds **19-23**, along with the α-bromoacryloyl derivative of desformyldistamycin A (**9**) and the corresponding two-pyrrole analogue **18,** were evaluated *in vitro* for their inhibitory effects on the proliferation of both mouse L1210 and human K562 leukaemia cells (Table **3**).

Table 3. ***In Vitro*** **Antiproliferative Activities of Compounds 9 and 18-27 Against Murine L1210 and Human K562 Leukemia Cell Lines**



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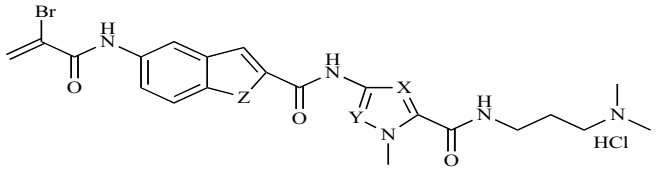


X=Y=CH, Z=NCH_3; **19**
X=Y=CH, Z=O; **20**
X=Y=CH, Z=S; **21**
X=N, Y=CH, Z=NCH_3; **22**
X=N, Y=CH, Z=O; **23**
X=N, Y=CH, Z=S; **24**
X=CH, Y=N, Z=NCH_3; **25**
X=CH, Y=N, Z=O; **26**
X=CH, Y=N, Z=S; **27**

Compound	IC_{50} (nM)	
	L1210	K562
9	79.6±22.4	n.d.
18	1300±76	n.d.
19	76.5 ± 24.2	493 ± 11.6
20	79.8 ± 34.8	436 ± 91.5
21	44.4 ± 25.7	306 ± 27.4
22	25.5 ± 8.2	78.7 ± 13.7
23	66.4 ± 12.8	263 ± 32.1
24	41.7 ± 11.5	196 ± 7.07
25	18.5 ± 5.4	55.2 ± 20.1
26	42.3 ± 13.1	133 ± 11.5
27	40.1 ± 10.1	227 ± 49.9

IC_{50}= 50% inhibitory concentration represents the mean ±SD from dose-response curves of at least three experiments.

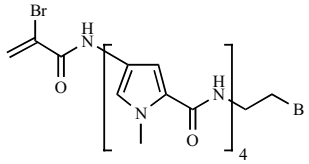
n.d= not determined.

The derivatives **19-27** showed an activity ranging between 20 and 80 nM against murine L1210 leukaemia cell line, while for the human K562 leukaemia cell line the IC_{50} values ranged 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. The α-bromoacryloyl di-pyrrole analogue **18** showed a 16-fold reduced cytotoxicity than the corresponding tri-pyrrole homologue **9** (IC_{50} = 1300 and 80 nM, respectively).When tested on L1210 cells, all the synthesized compounds **19-27** resulted from 16 to 72-fold more active than bis-pyrrole counterpart **18**, while several agents showed activity higher than or comparable with that of the α-bromoacryloylamido distamycin derivative **9**. The greatest potency and broadest spectrum of activity against both human and murine leukemia cell lines were exhibited by derivatives **22** and **25**, with IC_{50} values ranging from 18 to 80 nM, while compounds **19** and **20** exhibited lowest activity. The replacement of the pyrrolic ring joined to the α-bromoacryloyl moiety of the reference compound **18** by benzoheterocycles, such as *N*-methyl indole, benzofuran or benzothiophene, to generate derivatives **19, 20** and **21**, respectively, led to an improvement in terms of cytotoxic activity.

α-Bromoacryloyl Distamycin Derivatives Modified at the Amidino Moiety

The structure-activity relationship (SAR) of novel α-bromoacryloyl tetrapyrrolic distamycin A derivatives modified at the amidino moiety has been investigated by Cozzi *et al.* [21]. In this series of derivatives **28-41**, the amidino moiety was replaced by various groups, which included basic amidino-derived analogues of different lipophilicity and bulk, and non-basic groups of different nature. Table **4** data clearly showed that the potent cytotoxicity of the parent amidino derivative PNU-151807 (**10**) was fully maintained

Table 4. ***In Vivo*** **and** ***In Vitro*** **Activity of Compounds 10 and 28-41 Against L1210 Murine Leukemia**



Compound	B	*In Vitro* L1210	*In Vivo* [a]	
		IC_{50} (nM±S.E.)	O.D. (mg/Kg)	%T/C
10 (PNU-151807)	$C(NH)NH_2 \cdot HCl$	6.3±1.34	1.56	200
28	$C(NCH_3)NH_2 \cdot HCl$	2.67±0.78	3.13	150
29	$C(NCH_3)NHCH_3 \cdot HCl$	1.86±0.18	1.56	258
30	$C(NH)N(CH_3)_2 \cdot HCl$	1.48±0.84	n.d.	n.d.
31	C-Imidazolin-2-yl·HCl	2.46±0.52	n.d.	n.d.
32 (PNU-166196)	$NHC(NH)NH_2 \cdot HCl$	1.85±0.17	1.56	196
33	$CH_2NHC(NH)NH_2 \cdot HCl$	20.70±8.70	n.d.	n.d.
34	$(CH_2)_2NHC(NH)NH_2 \cdot HCl$	7.20±3.20	n.d.	n.d.
35	$NHC(NCH_3)NHCH_3 \cdot HCl$	1.26±0.38	n.d.	n.d.
36	NH-C-Imidazolin-2-yl·HCl	3.81±0.89	n.d.	n.d.
37	$NHCONH_2$	48.90±9.0	n.d.	n.d.
38	$C(NOH)NH_2$	8.58±1.55	6.25	186
39	$C(NCN)NH_2$	4.13±0.27	3.13	157
40	$CONH_2$	9.00±2.43	6.25	169
41	CN	18.1±2.27	12.5	157

IC_{50}= 50% inhibitory concentration as the mean ±SE from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

[a] For *in vivo* studies L1210 cells were injected iv at day 0 and mice were treated iv the day after tunor inoculum.

O.D= optimal dose; optimal non toxic dose<LD_{10} (weight loss <20% rispect to the starting weight)

%T/C= median survival time of treated vs. untreated mice x 100.

n.d.=not determined.

not only by basic amidino-derived compounds of different lipophilicity and bulk, such as N-methylamidine (**28**), N,N'-dimethylamidine (**29**), N,N-dimethylamidine (**30**), 2-imidazoline (**31**), guanidine (**32**, PNU-166196) and guanidino modified derivatives (**35** and **36**), but also by non-basic amidino-derived compounds such as amidoxime (**38**) and cyanoamidine (**39**), and even by non-basic, non-amidino-derived amide **40**. Both nitrile (**41**) and ureido (**37**) derivatives showed a modest decrease of activity. Alkylamidino and guanidino derivatives appear even more potent cytotoxics than the parent amidino compound [21,22].

By the synthesis of guanidino derivatives **33** and **34**, the distance between the polypyrrole backbone and the positive charge has been evaluated. The data reported in Table **4** showed that the progenitor guanidino derivative **32** resulted more active than analogues **33** and **34**, wherein the distance between the positive charge of the guanidino moiety from the polypyrrole frame was increased by addition of one and two methylene units (compounds **33** and **34**, respectively).

These results not only indicated that the amidino moiety was not an absolute requirement for activity, but also a lack of any correlation between the basicity of the amidine-like structure and cytotoxicity.

Moreover, the *in vivo* antileukemic activities appeared equivalent to, or even better than, that of the parent PNU-151807. In particular due to a favorable myelotoxicity/cytotoxicity ratio, the guanidino derivative **32** (brostallicin, PNU-166196) was selected for further extensive evaluation *in vivo* on murine solid tumors and human xenografts.

Compound **32** resulted 20-fold more active than tallimustine in inducing apoptosis in A2780 human ovarian carcinoma cells, circumvents the *in vitro* and *in vivo* resis-

tance to alkylating agents and topo I inhibitors. PNU-166196 also showed an outstanding myelotoxicity/cytotoxicity ratio, being its mean IC_{50} against a series of tumor cells about eighty times lower than its IC_{50} on human CFU-GM hematopoietic progenitors cells. Compound **32**, as the parent PNU 151807, appears unreactive in DNA alkylation assays but it was found to bind reversibly to the same DNA minor groove regions recognized by Tallimustine and Distamycin A.

PNU-166196 was found active *in vitro* against a broad spectrum of tumor cell lines and *in vivo* on tumour xenografts, with an improved therapeutic window over the other alkylating agents tested so far (Bizelesin, Adozelesin, Carzelesin and Tallimustine) [23].

PNU-166196 has an unique pharmacological profile, in fact it reacts *in vitro* with glutathione (GSH) in the presence of glutathione *S*-transferase (GST), but instead of giving inactivation on the compound as one might expect, this reaction increases the cytotoxicity of Brostallicin [24]. This compound is one of the first anticancer agents whose activity is enhanced by high GSH contents in contrast with the loss of activity observed for many drugs in tumour expressing high levels of GSH [25].

Both for amidino and guanidino derivatives PNU-151807 (**10**) and PNU-166196 (**32**), respectively, the antiproliferative activity decreased reducing the length of polypyrrolic frame. In fact, the three pyrrole unit derivatives **9** and **42** are about one order of magnitude less cytotoxic than the corresponding four pyrrole congeners PNU-151807 (**10**) and PNU-166196 (**32**), respectively. An identical trend of cytotoxicity occurs with the α-bromo derivatives with two and one pyrrolic units (compounds **18**, **43** and **44**), which are therefore devoid of significant activity (Table **5**).

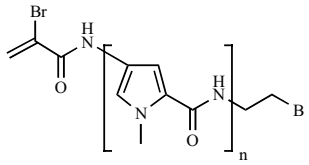
Beria *et al.* have also reported the *in vitro* and *in vivo* activities of a small series α-bromoacrylamido monopyrrole derivatives of low molecular weigh, in which the ethyl amidino terminus of **44** was replaced with a phenyl (compound **45**) or the amidino moiety was replaced by the non basic cyano group (compound **46**). Both these derivatives are characterized by a relevant antiproliferative activity *in vitro* but inactive *in vivo*, possibly because of the metabolic liability of the α-bromoacrylic moiety (Table **6**) [26].

In particular, compounds **45** and **46**, lacking of a strong basic moiety were potent cytotoxics, while analogue **44** bearing the strong basic amidino moiety was not. This suggests the existence of an active transport mechanism for distamycin derivatives PNU-166196 (**32**) and PNU-151807 (**10**), characterized by strong basic guanidino and amidino moiety, respectively.

In the same article, Beria *et al.* have reported the biological activity of some non-pyrrolic α-bromoacrylic anilide derivatives (compounds **47**-**50**) in which the distamycin frame was absent. The anilide (**47**), p-fluoroanilide (**48**) and N, N'-dimethylaminoanilide (**49**) derivatives showed a significant cytotoxicity, closely comparable with that of **45**, while the p-amidinoanilide derivative **50** showed the same inactivity of basic amidino derivative **44**. As for the monopyrrolic derivatives **45** and **45**, compounds **47-49** are devoid of antileukemic activity *in vivo*, accompanied also by a substantial lack of significant toxicity, particularly evident for compound **48**, in spite of their relevant cytotoxicity *in vitro*. A possible explanation for *in vivo* inactivity could be a massive metabolic degradation, which may depend upon the presence of the same α-bromoacrylic moiety which is responsible for the cytotoxicity and which is the sole reactive moiety of α-bromoacrylic anilides (Table **7**).

The inactivity of compounds **44** and **50** could be explained by the lack of cellular membrane permeability de-

Table 5. ***In Vitro*** **Activity of Amidine and Guanidine Derivatives 9, 10, 32 and 42-44 Against L1210 Murine Leukemia**

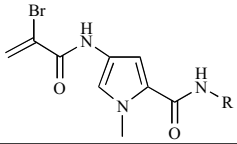


Compound	n	B	*In Vitro* IC_{50} (nM±S.E.)
10 (PNU-151807)	4	$C(NH)NH_2 \cdot HCl$	6.3±1.34
32 (PNU-166196)	4	$NHC(NH)NH_2 \cdot HCl$	1.85±0.17
9	3	$C(NH)NH_2 \cdot HCl$	79.6±22.4
42	3	$NHC(NH)NH_2 \cdot HCl$	27.0±3.1
18	2	$C(NH)NH_2 \cdot HCl$	1300
43	2	$NHC(NH)NH_2 \cdot HCl$	>500
44	1	$C(NH)NH_2 \cdot HCl$	>2500

IC_{50}= 50% inhibitory concentration as the mean ±SE from dose-response curves of at least three experiments.

Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

Table 6. *In Vivo* and *In Vitro* Activity of Compounds 44-46 Against L1210 Murine Leukemia



Compound	R	*In Vitro* L1210	*In Vivo* [a]	
		IC_{50} (nM±S.E.)	O.D. (mg/Kg)	%T/C
44	$CH_2CH_2C(NH)NH_2 \cdot HCl$	>2500	1.56	200
45	C_6H_5	43	25	100
46	CH_2CH_2CN	51.9	40	100

IC_{50}= 50% inhibitory concentration from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

[a] For *in vivo* studies L1210 cells were injected iv at day 0 and mice were treated iv the day after tunor inoculum.

O.D= optimal dose; optimal non toxic dose<LD_{10} (weight loss <20% rispect to the starting weight)

%T/C= median survival time of treated vs. untreated mice x 100.

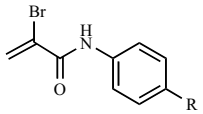
n.d.=not determined.

pending upon the ionization of the molecules due to the presence of strongly basic amidino moiety. The mild basicity of dimethylanilino derivative **49** should avoid full ionization of the molecule at pH 7.4, thus allowing cellular membrane permeability and explaining *in vitro* activity.

The positive role played by some modifications of the amidino moiety by other amidino-like or non basic-amidino-like moieties of different physico-chemical features was also confirmed in the case of α-bromoacryloyl pyrazole, imidazole and benzoheterocyclic derivatives of distamycin A (compounds **51-64**). In this new series of compounds the amidino moiety of compounds **11-16** was replaced by basic (pK_a values for the N-methylamidine and N, N-dimethylamidine residues are 12.37 and 12.68, respectively) and non-basic (pK_a=0.256 for the cyanoamidine fragment) groups.

Table **8** showed the antiproliferative activity of tested compounds on L1210 murine leukemia cells and L1210 leukemic cell line resistant to doxorubicin (DX). The results showed that these compounds display low resistance index values (R.I.=ratio between IC_{50} values on resistant cells and sensitive cells), ranging from four to eight, showing that neither the modification on the amidino moiety nor the heterocycle joined to the α-bromoacryloyl moiety were involved in the resistance mechanism.

Table 7. *In Vivo* and *In Vitro* Activity of Compounds 47-50 Against L1210 Murine Leukemia



Compound	R	*In Vitro* L1210	*In Vivo* [a]	
		IC_{50} (nM)	O.D. (mg/Kg)	%T/C
47	H	36.8	12.5	117
48	F	58.6	>50	100
49	$N(CH_3)_2 \cdot HCl$	54	25	100
50	$C(NH)NH_2 \cdot HCl$	>5000	n.d.	n.d.

IC_{50}= 50% inhibitory concentration from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

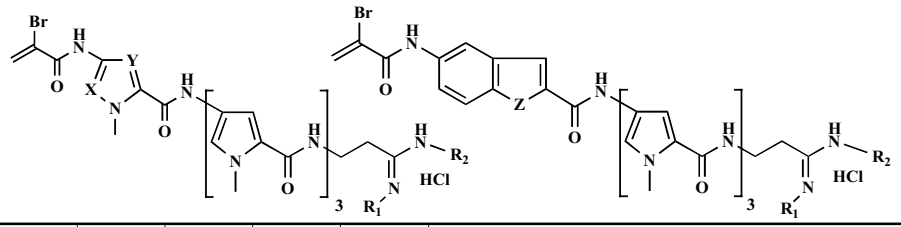
[a] For *in vivo* studies L1210 cells were injected iv at day 0 and mice were treated iv the day after tunor inoculum.

O.D= optimal dose; optimal non toxic dose<LD_{10} (weight loss <20% rispect to the starting weight).

%T/C= median survival time of treated vs. untreated mice x 100.

n.d.=not determined.

Table 8. ***In Vitro*** **Activity and Resistance Index (R.I.) of Derivatives 10-16 and-51-64 Against L1210 and L1210 Murine Leukemia Cells Resistant to the Doxorubicin (DX)**



Compound						IC_{50} (nM±S.E.)		R.I[a]
10	X	Y	Z	R_1	R_2	L1210	L1210/DX	
11	N	CH	/	H	H	13.3±0.54	93.1±17	7
12	CH	N	/	H	H	46.9±13	726.9±6.9	15.5
13	/	/	NH	H	H	4.1±1.3	35.8±5.6	8.7
14	/	/	NMe	H	H	2.4±0.39	43.6±1.5	18
15	/	/	O	H	H	6.1±0.93	39.0±2	6.4
16	/	/	S	H	H	14.3±5.89	48.3±2	3.4
51	N	CH	/	H	Me	10.3±0.26	38.6±3	3.8
52	N	CH	/	Me	Me	25.6±3.75	115.7±3.4	4.5
53	N	CH	/	H	CN	7.7±0.34	41.0±4	5.3
54	CH	N	/	H	Me	155.1±52.9	970.0±11	6.3
55	CH	N	/	Me	Me	59.7±15.6	200.2±4.1	3.4
56	CH	N	/	H	CN	186±11.48	914.8±15.2	4.9
57	/	/	NH	H	Me	4.5±0.25	23.5±7	5.2
58	/	/	NH	Me	Me	7.6±2.84	26.7±7	3.5
59	/	/	NMe	H	Me	4.8±0.49	38.3±1.11	7.9
60	/	/	NMe	Me	Me	2.8±0.36	15.4±2	5.6
61	/	/	O	H	Me	9.5±1.76	33.3±9	3.6
62	/	/	O	Me	Me	6.3±0.62	38.6±1	6.1
63	/	/	S	H	Me	10.5±1.48	53.2±4	5.1
64	/	/	S	Me	Me	17.9±6.66	62.3±5	3.5

IC_{50}= 50% inhibitory concentration from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 and L1210/Dx cells.

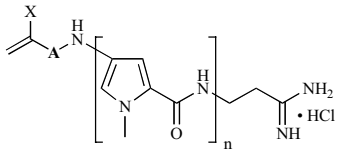
a: R.I (resistance index)= ratio between IC_{50} values on resistant cells and sensitive cells.

With the only exception of compounds **54** and **56** (IC_{50}=133 and 186 nM, respectively), all tested molecules exhibited strong growth inhibition activities on L1210 murine leukemia cells, with IC_{50} ranged between 2 and 80 nM. The neutral or positive role played by the modifications of the amidino moiety was also confirmed for the pyrazolic and benzoheterocyclic derivatives **51-53** and **57-64**, respectively. These compounds maintained or even improved the cytotoxicity of the amidine parent compounds **11** and **13-16**, respectively. The data indicate a lack of correlation between the basicity of the amidine-like structure and growth inhibition activity, where this latter only depends from the isosteric five-membered heterocyclic ring (N-methylpyrazole and N-methylimidazole) joined to the α-bromoacrylic moiety [27].

3. MECHANISM OF ACTION RELATED TO HALOGENOACRYLIC DERIVATIVES OF DISTAMYCIN A

By the synthesis of a series of α-bromo, α-chloro and α-fluoro acrylic derivatives of Distamycin A (compounds **9**, **67** and **68**, respectively) and the corresponding tetra-pyrrolic congeners (compounds **10**, **69** and **70**), the structure-activity

Table 9. ***In Vivo*** **and** ***In Vitro*** **Activity of Compounds 9, 10 and 66-70 Against L1210 Murine Leukemia**



Compound	n	A	X	*In Vitro* L1210	*In Vivo* [a]	
				IC_{50} (nM±S.E.)	O.D. (mg/Kg)	%T/C
9	3	C=O	Br	79.6±14	12.5	175
10 (PNU-151807)	4	C=O	Br	6.3±1.34	1.56	200
65	3	CH_2	Br	>1600	n.d.	n.d.
66	3	C=O	H	>50,000	n.d.	n.d.
67	3	C=O	Cl	96.8±24.2	12.5	117
68	3	C=O	F	>800	n.d.	n.d.
69	4	C=O	Cl	3.8±1.4	1.56	133
70	4	C=O	F	>700	n.d.	n.d.

IC_{50}= 50% inhibitory concentration as the mean ±SE from dose-response curves of at least three experiments. Drug sensitivity was determined after 48 h of continuous exposure against L1210 cells.

[a]For *in vivo* studies L1210 cells were injected iv at day 0 and mice were treated iv the day after tunor inoculum.

O.D= optimal dose; optimal non toxic dose<LD_{10} (weight loss <20% rispect to the starting weight)

%T/C= median survival time of treated vs. untreated mice x 100.

n.d.=not determined.

relationship indicated a key role of the reactivity of the α-halogenoacrylic moiety (Table **9**) [28].

While both α-bromo and α-chloro derivatives of the same four or three pyrrole units frame are substantially equipotent *in vitro*, the three pyrrole unit derivatives (compounds **9** and **67,** respectively) are about one order of magnitude less cytotoxic than the corresponding four pyrrole congeners **10** and **69**. The α-fluoroacrylic (compounds **68** and **70**), α-bromovinyl (compound **65**) and acrylamido (compound **66**) derivatives appear devoid of significant activity.

The reactivity of the α-halogenacrylic moiety, due to the low reactivity of the vinylic halogen, could be based on a first-step Michael-type nucleophilic attack to the double bond to furnish a reactive α-halogenoamido intermediate, which can undergo a further reaction of the no more vinylic halogen leading to a second nucleophilic substitution or to beta elimination as reported in Fig. (**3**). A mechanism of this kind, implying a Michael attack followed by a classical nucleophilic displacement of bromo substituent alpha to a carbonyl, was reported following the so-called Gabriel-Cromwell reaction [29].

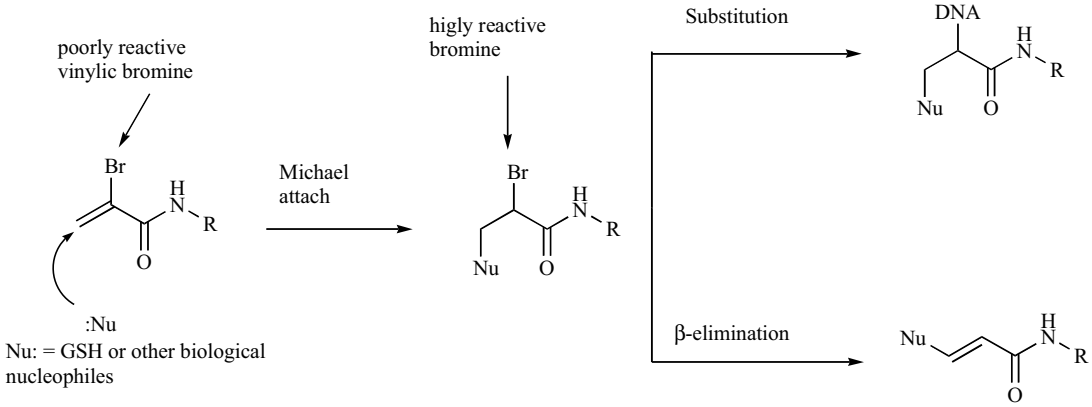


Fig. (3). Hypothesized mechanism of activation of α-bromoacrylamido derivatives by biological nucleophiles.

This hypothesis was supported by the loss of activity occurring when the carbonyl function alpha to the halogen was absent (compound **65**), making the Michael attack impossible. The inactivity of acrylic (**66**) and of α-fluoroacrylic (**68** and **70**) analogues could be explained by the reversibility of the Michael reaction when the further reaction was impossible (compound **66**) or difficult, being based on the bad leaving capability of the fluoro alpha to the carbonyl (**68** and **70**). The inactivity of acrylic derivative **66** confirms the requirement of a reactive halogen on the double bond.

To explain the role of GSH in the enhancement of the *in vitro* activity of PNU-151807 and on the basis of the electrophilic reactivity of its α-bromoacrylic moiety, we speculated that GSH, as an intracellular reactive nucleophilic species, could react with the α-bromoacrylamide moiety, leading to the formation of a highly reactive GSH complex representing the real effective agent of brostallicin activity, leading to alkylation of DNA nucleophilic functions.

In contrast to classical alkylating agents, which are less effective in tumor cells with high GSH/GST levels, the cytotoxic activity of brostallicin and PNU-151807 was higher in L1210/LPAM cells characterized by a 3-fold increase of GSH level in respect to the wild-type L1210 cell line [30].

4. HYBRID MOLECULES CONTAINING α-METHYLENE-γ-BUTYROLACTONES AND α-BROMOACRYLOYL BENZOHETEROCYCLIC MOIETIES

α-Methylene-γ-butyrolactone derivatives have attracted much attention over the years, since the α-methylene-γ-butyrolactone ring is an important functional structure in a wide range of natural products [31, 32], particularly cytotoxic sesquiterpene lactones such as 1-*O*-acetylbritannilactone **71** [33], methylenolactocin **72** [34], protolichesterinic acid **73** [35] and helenalin **74** [36]. It was soon determined that the structural requirement for the biological activities is mainly associated with the exocyclic, conjugated double bond (the O=C=C=CH_2 moiety), which acts as an alkylating agent *via* Michael-type reaction with cellular nucleophiles or cysteine residues of functional proteins to form covalent bonds [37].

Based on these considerations, our group had reported the preparation and biological evaluation of a novel series of conjugates **75-85** that have two moieties in their structures acting as Michael acceptors (Fig. (**4**)). In these hybrid molecules, the α-bromoacrylic derivative of benzoheterocyclic rings (compounds **86-89)**, such as indole, N-methyl indole, benzofuran and benzothiophene [16], was tethered, *via* a flexible ethylenediamino chain, to a pyrazole moiety linked

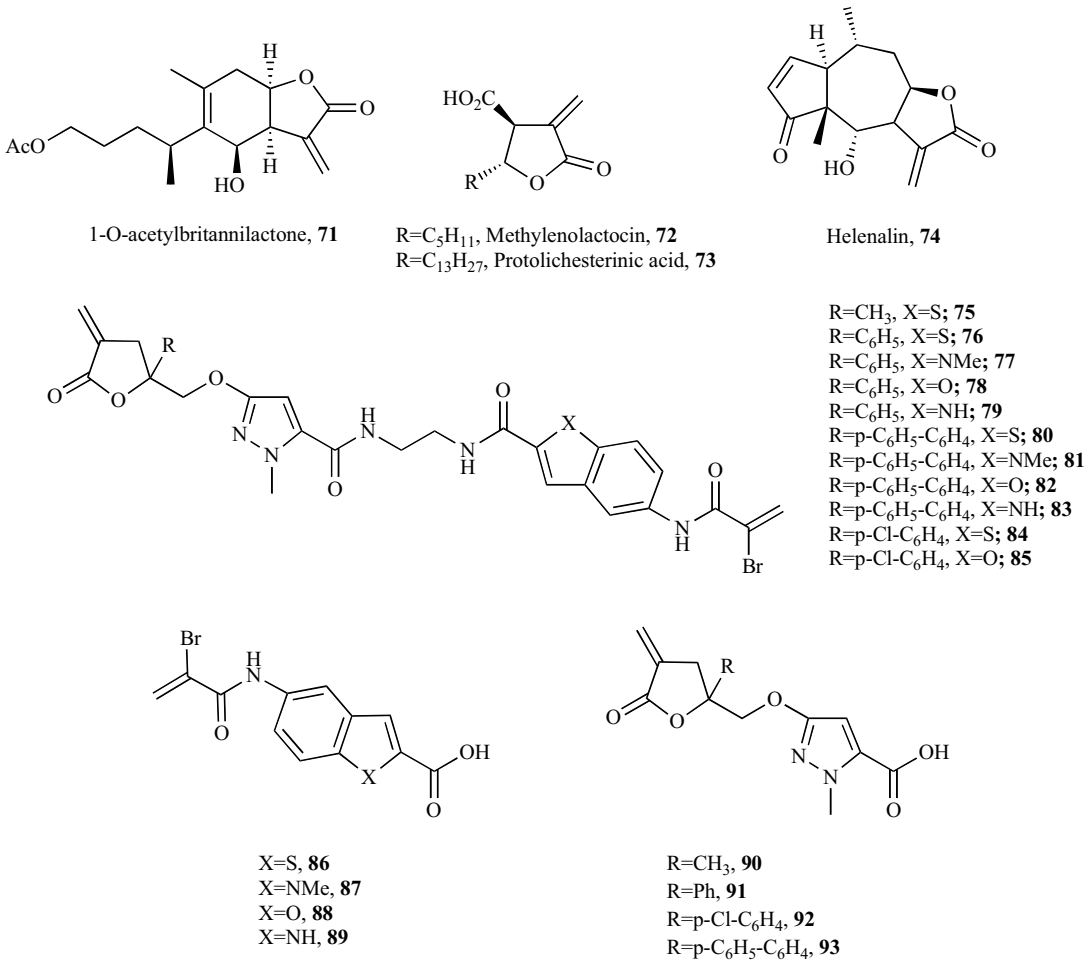


Fig. (4). Chemical structures of sesquiterpene lactones **71-74**, heterobifunctional compounds **75-85**, α-bromoacryloyl benzoheterocyclic amides **86-89** and α-methylene-γ-butyrolactones **90-93**.

to a -methylene-γ–methyl/aryl-γ-butyrolactone residue by a methylene unit (derivatives **90-93**) [38].

As evident from Table **10**, the growth inhibitory activity of each hybrid compound **75-85** proved to be much greater than that of the alkylating units tested alone against mouse L1210 and human K562 leukaemia cells.

Table 10. ***In Vitro*** **Activity of Hybrids 75-85 and Alkylating Units 86-93 Against the Proliferation of Murine L1210 and Human K562 Leukemia Cell Lines**

Compound	IC_{50} (μM)[a]	
	L1210	K562
75	0.84±0.14	1.02±0.09
76	0.29±0.06	0.27±0.04
77	0.05±0.01	0.08±0.00
78	0.31±0.08	0.42±0.17
79	0.85±0.03	0.63±0.08
80	0.29±0.01	0.38±0.01
81	0.28±0.01	0.36±0.06
82	0.27±0.02	0.41±0.01
83	0.36±0.05	0.48±0.03
84	0.26±0.01	0.41±0.01
85	0.55±0.03	0.71±0.09
86	4.50±0.70	5.00±0.14
87	2.07±0.24	3.37±0.17
88	2.95±1.34	7.25±0.35
89	4.30±0.50	5.60±1.32
90	>10	>10
91	>10	>10
92	>10	>10
93	>10	>10

[a]IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50% Data are expressed as the mean ± SE from the dose-response curves of at least three independent experiments.

The derivatives **75-85** showed an activity ranging between 50 and 800 nM against murine L1210 leukaemia cell line, while for the human K562 leukaemia cell line the IC_{50} values ranged between 80 and 1,000 nM. The greatest potency was shown by the compound **77**, constituted by the α-methylene-γ–phenyl-γ-butyrolactone **91** and the α-bromoacrylamido-N-methylindole derivative **87**, with IC_{50} values of 55 and 82 nM against L1210 and K-562 cell lines, respectively.

A significant increase in antiproliferative activity was observed when the γ-methyl group of **75** was replaced with a phenyl, to gave compound **76**, which exhibited the same activity against both cell lines, but resulted 3- and 4-fold more active than **75** against L1210 and K562 cells, respectively. The good antiproliferative activity of **76** implies that a lipophilic and bulky substituent at the γ-position of the lactone increases the cytostatic potency.

Using the human leukemia HL-60 cell line, a selected series of compounds (**77**, **78**, **80** and **84**) was found to induce morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death. Finally, our results point to the fact that the compounds investigated induce apoptosis *via* activation of caspase.

5. HYBRIDS PREPARED COMBINING BENZO[4,5] IMIDAZO[1,2-D][1,2,4]THIADIAZOLE AND BENZO-HETEROCYCLIC α-BROMOACRYLOYL AMIDES.

In recent years, several research groups have disclosed the use of benzo[4, 5]imidazo[1, 2-d][1, 2, 4]thiadiazoles ([1, 2, 4]BTHD's) as inhibitors targeting cysteine residue of biomolecules [39]. It is noteworthy to note that the [1, 2, 4] BTHD system displayed a lack of reactivity towards other nucleophiles such as amines and alcohols [40]. An appropriate C-3 substituent (R) in the tricyclic [1, 2, 4]BTHD can enhance both enzyme affinity and reactivity [41, 42].

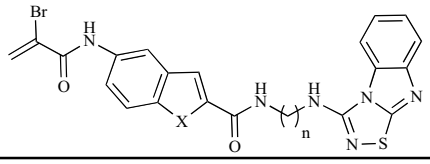
Following the finding that both benzo[4, 5]imidazo[1, 2-*d*][1, 2, 4]thiadiazole and α-bromoacryloyl moiety may act as thiol trapping agents, our group has recently reported the synthesis and biological activity of a series of hybrids **94-98** incorporating these two moieties in their structures [43]. These hybrid molecules are constituted by the α-bromoacrylic derivative **86-89** tethered *via* a flexible propyl/hexyldiamino spacer, to the C-3 position of the [1, 2, 4]BTHD nucleus.

All these hetero-bifunctional compounds were highly cytotoxic against the human myeloid leukemia cell lines HL-60 and U937 (IC_{50} 0.24-1.72 μM), resulting significantly more potent than the alkylating units tested alone (Table **11**). Similar results were obtained with the human melanoma cell line SK-MEL-1, however the IC_{50} values were higher than that on human myeloid cells.

The relationship between the heteroatom in the benzoheterocycle and antiproliferative activity revealed that *N*-unsubstituted indole derivatives **94** and **95** were the most active. In the series of derivatives **95-98** characterized by the same alkyl chain (n=6), the greatest potency was exhibited by compound **95** with IC_{50} values of 0.55, 0.40 and 3.02 μM against HL-60, U937 and SK-MEL-1 cells, respectively. A progressive decrease in antiproliferative activity was observed replacing indole with N-methylindole to end with benzofuran and benzothiophene (compounds **95**, **96**, **97** and **98**, respectively).

On human myeloid leukaemia HL-60 cells, we observed that these compounds suppress survival and proliferation by triggering morphological changes and internucleosomal DNA characteristic fragmentation characteristic of apoptotic cell death. The apoptosis induced by these derivatives was mediated by caspase-3 activation and was also associated to an early release of cytochrome *c* from the mitochondria.

Table 11. Effects of Hybrid Compounds 94-98 and α-Bromoacryloyl Benzoheterocyclic Amides 86-89 on the Growth of HL-60, U937 and SK-MEL-1 Cells Cultured



n=3, X=NH, **94**
n=6, X=NH, **95**
n=6, X=NMe, **96**
n=6, X=O, **97**
n=6, X=S, **98**

Compound	IC_{50} (μM)		
	HL-60	U937	SK-MEL-1
94	0.29±0.03	0.24±0.03	2.09±0.39
95	0.55±0.18	0.40±0.11	3.02±0.62
96	0.84±0.06	0.89±0.06	4.50±0.49
97	1.72±0.65	0.74±0.12	8.95±0.45
98	1.24±0.12	0.91±0.05	8.04±0.78
86	>10	>10	>10
87	>10	>10	>10
88	>10	>10	>10
89	>10	>10	>10

The data shown represent the mean (±S.E.M.) of three independent experiments with three determinations in each.

CONCLUSIONS

In this review article we have evidenced that all heterobifunctional compounds between α-bromoacryloyl amides and known antitumor agents (distamycin A derivatives, α-methylene-γ-butyrolactones and BTHD) demonstrated a biological activity significantly superior to that of both alkylating units alone. Although for several hybrid compounds (such as conjugate obtained linking α-bromoacryloyl amides with α-methylene-γ-butyrolactones or BTHD) the data do not allow the identification of the molecular target(s), preliminary experiments sustain the concept that these compounds retain low ability to alkylate DNA also at high concentration (10 μM). We have observed that all α-bromoacryloyl derivatives cited in this review article exerted their main action through interactions with cysteine residues of proteins, corresponding to GSH and caspase-3 for brostallicin and α-bromoacryloyl amides/α-methylene-γ-butyrolactones or BTHD hybrids, respectively.

The hybrid molecules prepared combining a BTHD or a α-methylene-γ-butyrolactones with different benzoheterocyclic α-bromoacryloyl amides suppress proliferation of HL-60 cells by triggering morphological changes and internucleosomal DNA fragmentation, which are well known features of apoptotic cell death. Further investigations are necessary to determine the detailed pathway of programmed cell death by these compounds.

Brostallicin is currently in phase II clinical trials versus doxorubicin as first-line single-agent chemotherapy in patients with advanced or metastatic soft tissue sarcoma, a cancer of the supporting tissue of the body. Data in more than 200 patients treated in phase I/II clinical trials reveal evidence of activity in patients with refractory cancer and patient/physician-friendly dosage and administration. Brostallicin may ultimately be useful in combination with standard chemotherapy and newer, targeted cancer therapies.

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