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A new class of cytotoxic DNA minor groove binders: α-halogenoacrylic derivatives of pyrrolecarbamoyl oligomers

Paolo Cozzi

Pharmacia, Department of Chemistry, Discovery Research Oncology, Viale Pasteur 10, 20014 Nerviano, Milan, Italy

Abstract

DNA minor groove binders represent a class of cytotoxic antitumor agents whose DNA sequence specificity may lead to a high selectivity of action. Tallimustine, benzoyl nitrogen mustard derivative of distamycin A, showed excellent antitumor activity in preclinical tests but also a severe myelotoxicity. Novel nitrogen mustard derivatives of distamycin showing improved activity profile were recently identified. In particular, cinnamic nitrogen mustard and cinnamic ethyl half-mustard analogs of tallimustine showed increased potency and more favorable cytotoxicity/myelotoxicity ratio. However a series of α-halogenoacrylamido derivatives of distamycin-like frames showed an activity profile substantially improved in comparison to tallimustine. In particular PNU-166196, α-bromo-acrylamido derivative of four pyrrole distamycin-like frame ending with a guanidino moiety, showed high cytotoxic potency even on tumor cell lines resistant to alkylating agents and camptothecin, broad antitumor activity and myelotoxicity dramatically reduced in comparison to tallimustine. This compound was found to bind to minor groove TA-rich sequences but appeared unreactive in classical in vitro DNA alkylation assays. With respect to the apparent lack of DNA alkylation we speculated that an intracellular reactive nucleophilic species, e.g. glutathione (GSH), could activate the reactivity of the compound leading to alkylation of DNA in vivo. Recent evidence of both covalent interaction of PNU-166196 with plasmidic DNA in the presence of GSH and of enhanced cytotoxicity in tumor cells characterized by high levels of GSH were obtained. PNU-166196, in view of its excellent activity profile and its outstanding favorable cytotoxicity/myelotoxicity ratio, was selected for clinical development and is undergoing phase I studies.

Keywords: Minor groove binders; Distamycin; Tallimustine; α-Halogenoacrylamides; Glutathione

1. Introduction

The putative mode of action of many antitumor agents involves DNA damage, either by direct binding of the drug to DNA or to DNA-binding proteins. However, most of the DNA-interacting agents have only a limited degree of sequence specificity, which implies that they may hit all the cellular genes.

DNA minor groove binders, among which the derivatives of distamycin A (DST) play an important role, could provide a significant improvement in cancer management increasing gene specificity, due to high selectivity of interaction with thymine-adenine (TA) rich sequences [1].

DST, an antibiotic characterized by an oligopeptidic pyrrolic frame ending with an amidino moiety [2], Fig. 1, binds reversibly to DNA minor groove with high selectivity for TA-rich sequences containing at least four TA base pairs [3]. Due to the high selectivity of DNA interaction, DST was used in the recent past as a DNA sequence-selective vector of alkylating functions. In particular, tallimustine (TAM) [4], Fig. 1, a benzoic acid nitrogen mustard (BAM) derivative of DST, that binds selectively to DNA as DST does [5,6], shows excellent cytotoxic and antitumor activity [4].

TAM was the first and until very recently the sole DST-derived cytotoxic that was clinically developed. However TAM showed severe myelotoxicity [7] that probably impaired the attainment of effective therapeutic doses [8,9] and its phase II clinical development was discontinued.

Nevertheless TAM has represented an important model for the design of new cytotoxics in which a moiety of mild chemical reactivity, such as the BAM moiety in the case of TAM, is tethered to a DNA binding-frame derived from DST.

E-mail address: paolo.cozzi@eu.pnu.com (P.cozzi).

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Derivatives of DST aroused particular interest because of their oligopeptidic nature, which suggested the possibility of modulating the DNA binding capability by varying the number and the characteristics of the azole carbamoyl units, (Fig. 2). The hydrogen bonding between amidic protons of DST and DNA plays a key role for binding. The increased multiplicity of this bonding leads to a tighter DNA interaction [10]. Therefore it is not surprising that in the case of distamycin-derived nitrogen mustards the cytotoxicity increases about one order of magnitude every additional amidic unit present, to a maximum of five [11].

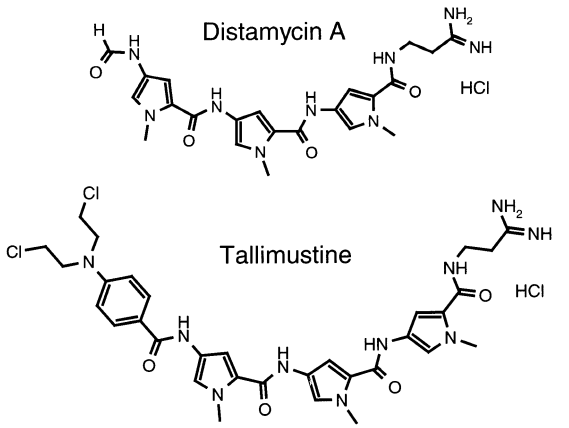


Fig. 1. Distamycin A and its benzoyl nitrogen mustard tallimustine [28]. Distamycin A: $IC_{50} = 10.000$ nM against L1210 murine leukemia. Tallimustine: $IC_{50} = 68.5$ nM against L1210 murine leukemia.

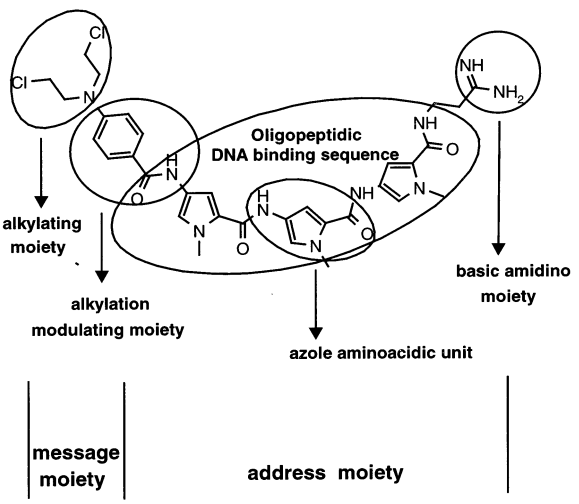


Fig. 2. The different functional moieties of tallimustine.

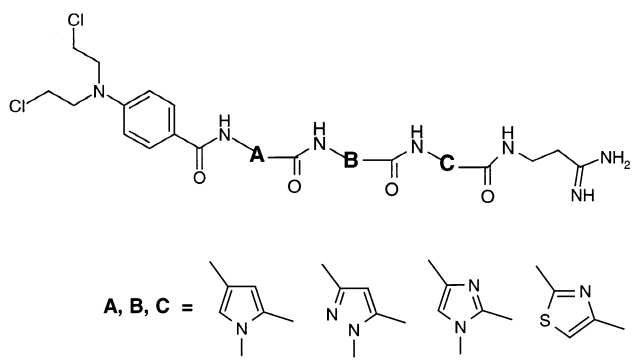


Fig. 3. Representative examples of isosteric tallimustine analogs [17,35].

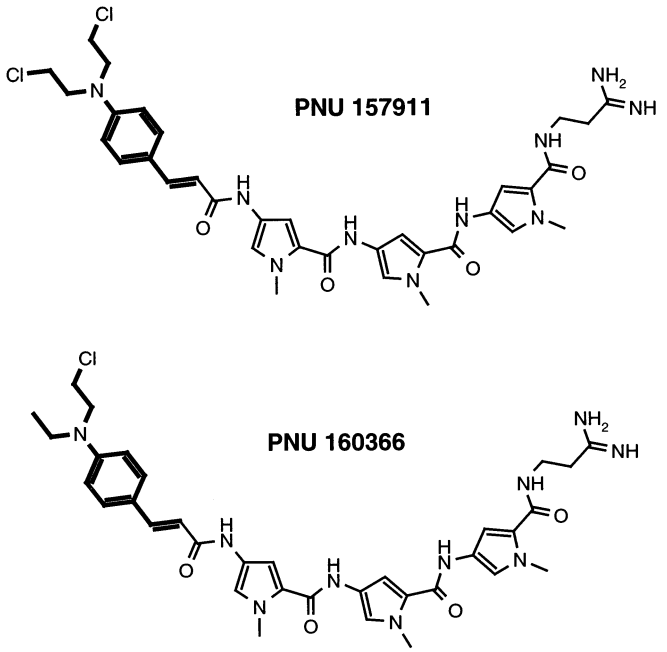
In the recent past different groups, including ourselves, described various nitrogen mustard derivatives of DST-like frames in which one or more pyrrole units were replaced by other pentatomic heteroaromatic rings, mainly imidazole or pyrazole [12–18], Fig. 3. This approach was based partly on the concept of lexitropsin, hypothesized independently by Dickerson and Lown [19–21], and partly on the concept of isosteric replacement of the pyrrole units by pyrazole rings first hypothesized by Denny [22]. Several of these isosteric mustards showed activities comparable with those of TAM, however these compounds apparently did not improve the activity profile of TAM [11].

Novel mustard derivatives of DST showing improved activity profile have been recently identified by our group. Cinnamic nitrogen mustard PNU-157911, a vinylog of TAM, and cinnamic ethyl half-mustard PNU-160366, Fig. 4, show high cytotoxicity, very good in vivo activity, and more importantly, a cytotoxicity/myelotoxicity ratio markedly improved in comparison to TAM, where this ratio is about unity [23–25]. PNU-160366 is characterized by being a one-arm mustard, whose activity underlines a mechanistic diversity from classical nitrogen mustards. In fact in the case of the classical nitrogen mustards the corresponding half-mustards are substantially non-cytotoxic [26], possibly due to the impossibility of crosslinking the two DNA strands [27]. These two cinnamic mustard derivatives represent in a sense the final evolution of the close rationale that led to TAM.

2. α-Halogenoacrylamido distamycin derivatives

Mongelli and coworkers some years ago described cytotoxics showing an α-bromo or α-chloro acrylamide moiety linked to DST or DST-like analogs. In particular PNU-151807, the α-bromo-acrylamido derivative of the four-pyrrole DST homolog, was found to have significant cytotoxicity and in vivo activity [28], (Fig. 5).

Recently, by using DNA footprinting analysis, PNU-151807 was found to bind reversibly to the same DNA minor groove regions recognized by TAM and DST but was found to be unreactive in classical in vitro DNA alkylation assays [29], at variance not only with TAM, but also with other cytotoxic minor groove binders. Apparently therefore PNU-151807 is a minor groove binder unable to alkylate DNA.



COMPOUND	MYELOTOXICITY Human CFU-GM[a] IC_{50} ng/mL	CYTOTOXICITY Tumor Cells[b] IC_{50} ng/mL	*In vitro* Cytotoxicity/ Myelotoxicity Ratio
PNU-157911	1680	70	**24**
PNU-160366	2109	175	**12**
TALLIMUSTINE	290	330	0.9

a Human colony-forming units - granulocytes-monocytes hematopoietic progenitor cells ;
b mean IC_{50} values of ten tumor cell lines.

Fig. 4. Cinnamic mustard and half-mustard distamycin derivatives: cytoxicity/myelotoxicity ratio.

An important point is that while α-bromo and α-chloro-acrylamido derivatives of this class show relevant cytotoxicity, α-fluoro-acrylamido and acrylamido derivatives appear substantially devoid of activity [30], Table 1. These data suggest a key role of the reactivity of the α-halogenoacrylic moiety for cytotoxicity, in spite of the fact that active compounds of this series were found unreactive in in vitro DNA alkylation assays.

Our hypothesis, from a purely chemical standpoint, is that the reactivity of the α-halogenoacrylic moiety, due to the low reactivity of the vinylic halide, should be based on a first-step Michael-type attack, followed by the further reaction of the no longer vinylic halide, leading to a second nucleophilic substitution or alternatively to β-elimination. A mechanism of this kind corresponds to the so-called Gabriel–Cromwell reaction [31,32], (Fig. 6).

Our hypothesis is supported by the experimental evidence of a dramatically different reactivity toward nucleophilic attack of α-bromoacrylamido and of α-fluoroacrylamido moieties [33]. The former, stable in aqueous alkaline conditions, reacts with primary and secondary amines undergoing double nucleophilic substitution, and with the imidazole ring, in the presence of K_2CO_3 as a base, undergoing nucleophilic substitution and β-elimination, while the latter gives no reaction in the same or harder conditions. The α-chloroacrylamido moiety shows an intermediate reactivity, Figs. 7 and 8.

The inactivity of acrylic and of fluoroacrylic analogs could be explained by the fact that a further attack by DNA nucleophilic functions is impossible in the case of

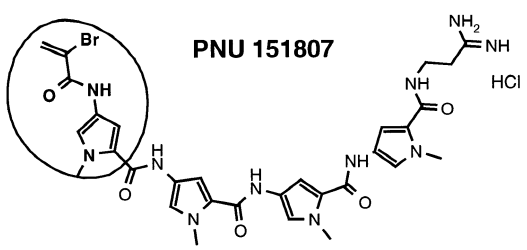
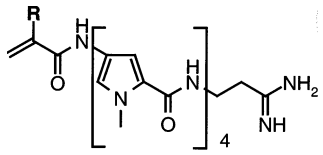


Fig. 5. PNU-151807: the lead of distamycin-derived α-halogenoacrylamides [28]. PNU-151807: IC_{50} (L1210) = 4.7 ng/ml. Tallimustine: IC_{50} (L1210) = 50.3 ng/ml.

Table 1
The role of the halogen on the activity of α-halogenoacrylamido distamycin derivatives: in vitro and in vivo activity (L1210 leukemia) [a] [30]



R	In vitro IC_{50} nM	In vivo	
		OD mg/kg	T/C %
Br	6.3 ± 1.3	1.56	200
Cl	3.8 ± 1.4	1.56	133
F	>700	Nd	nd
H	>3000	Nd	nd

[a] $IC_{50} = 50\%$ inhibitory concentration as the mean ± SE from dose–response curves of at least two experiments; drug sensitivity determined after 48 h of continuous exposure against L1210 cells; for in vivo studies cells were injected i.v. and mice were treated i.v. the day after tumor injection; OD optimal (non-toxic) dose $< LD_{10}$; T/C median survival time of treated versus untreated mice $\times 100$.

acrylic derivatives, or difficult in the case of fluoroacrylic derivatives, because of the poor leaving capability of the fluoro substituent.

3. Recent results

The relevant activity and the unusual mechanistic features of PNU-151807 prompted us to synthesize new halogenoacrylic derivatives of DST and congeners with the aim of optimizing their profile of activity and possibly helping to define their mechanism of action. Therefore we modified the length of the DST frame, replaced the amidino moiety with moieties of different features and replaced one or more pyrrole units with other imidazole or pyrazole units.

While both α-bromo and α-chloro derivatives of the same distamycin frame are substantially equipotent in vitro, the four-pyrrole unit derivatives are about one order of magnitude more cytotoxic than three-pyrrole unit congeners. The same decrease of activity occurs with α-bromo derivatives with two and one pyrrole units, which are therefore devoid of significant activity. Apparently, the five-pyrrole unit derivative does not follow completely this trend of cytotoxicity, Table 2.

This increased cytotoxicity is in agreement with the concept of tighter DNA binding depending on the increased multiplicity of interaction with the minor groove. The increase in the number of pyrrole units from three to five leads also to an increase of in vivo potency, while the survival time shows a peak in the case of the four-pyrrole derivative [30].

As far as the role of the strongly basic amidino moiety, typical of DST and TAM, is concerned, the activity of the parent amidino analog is fully maintained not only by basic amidino-like compounds of different lipophilicity and bulk, such as *N*-methylamidine, *N*-dimethylamidines, 2-imidazoline and by guanidine derivatives, but also by non-basic amidino derivatives such as amidoxime and cyanoamidine and by carboxyamide derivatives. Only the cyano derivative shows a modest decrease of activity [33], Table 3.

A lack of correlation between the basicity of the amidine-replacing moiety and cytotoxicity was already demonstrated by us in the case of nitrogen mustard derivatives of DST [34]. However, the high cytotoxic activity of the compounds of this series also occurs in the case of modifications which led in the mustard series to a significant decrease of activity.

The amidino moiety, due to its strong basic nature, implying total protonation in any biological condition, may play a key role both for DNA binding and cell or tissue bioavailability. The presence of non-basic moieties in highly active DST derivatives contrasts with the common opinion that electrostatic interaction between the cationic moiety and the negatively charged DNA phosphate residues may represent a main contribution to molecular recognition of DST and DST-like derivatives.

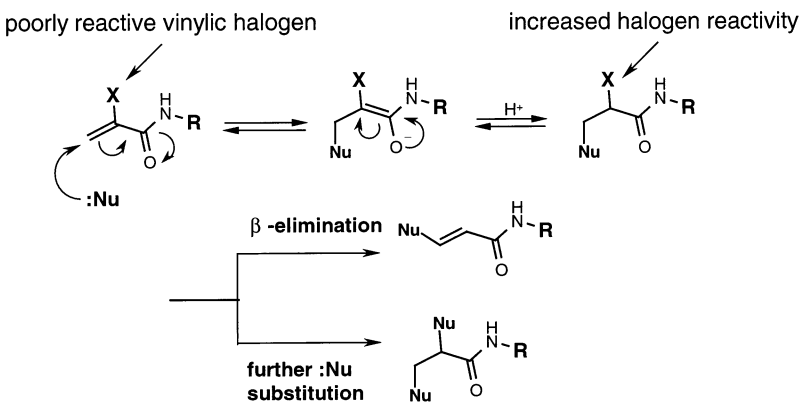


Fig. 6. Putative mechanism of nucleophilic attack to α-halogenoacrylic moiety.

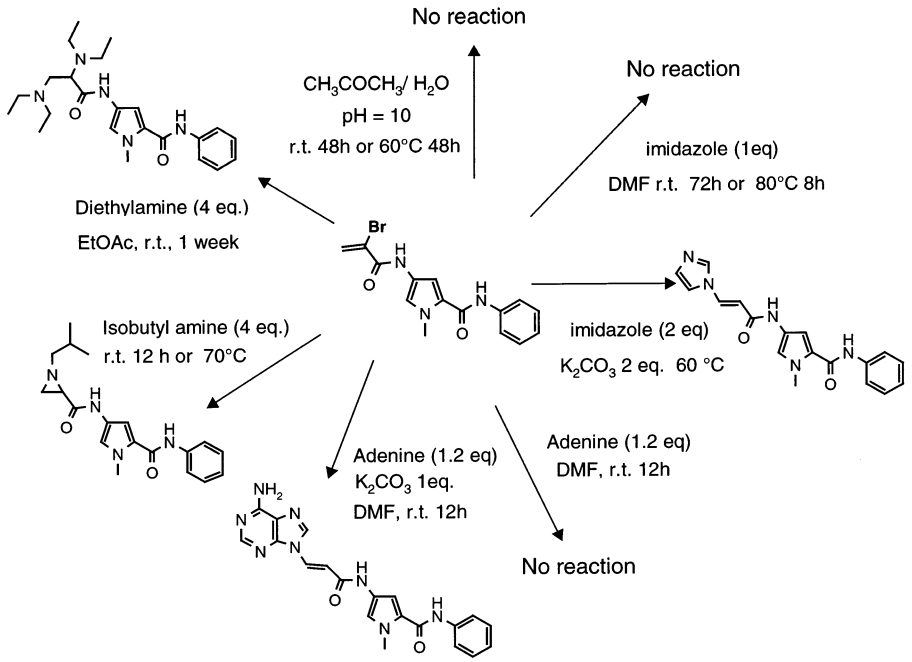


Fig. 7. Reactivity of α-bromoacrylamido moiety (α-bromoacrylamino-pyrrolecarboxyanilide).

Following the same rationale applied to nitrogen mustard derivatives, several α-bromoacrylic derivatives of DST-like frames in which one or more pyrrole units were replaced by other pentaatomic heteroaromatic rings, mainly imidazole or pyrazole, were synthesized.

Among the latter compounds, the analogs of PNU-151807 in which the *N*-methylpyrrole unit acylated by the α-bromoacrylic moiety was replaced by a pyrazole or imidazole unit, Fig. 9, showed antileukemic activity substantially equivalent to PNU-151807, while when the replacement of the pyrrole unit occurred far from the α-bromoacrylic moiety the activity was less satisfactory [35,36].

Some of these α-bromoacrylic derivatives of distamycin-like four azole oligomers, both of the pyrrole and of the imidazole/pyrazole series, appear significantly more potent than TAM and show a favorable myelotoxicity/cytotoxicity ratio. Therefore they were selected for further extensive antitumor investigation and the result of this process was the selection for clinical development of a compound of this class. PNU-166196, α-bromoacrylamido-tetrapyrrolecarbamoyl derivative ending with a guanidino moiety, Fig. 10, is presently undergoing phase I clinical trials.

PNU-166196 shows a broad spectrum of activity, circumvents resistance to alkylating agents and topoisomerase I inhibitors and shows an outstanding cytotoxicity/myelotoxicity ratio, its mean IC_{50} against a series of tumor cell lines being about eighty times lower than its IC_{50} on human CFU-GM hematopoietic progenitor cells [37], Table 4. Moreover PNU-166196 is 20 fold more active than TAM in inducing apoptosis in A2780 human ovarian carcinoma cells [38].

Finally this compound, as the parent PNU-151807, appears unreactive in DNA alkylation assays [37].

With respect to the apparent lack of DNA alkylation we speculated that an intracellular reactive nucleophilic species, e.g. glutathione (GSH), could perform a first-step Michael-type attack, which may be followed by a

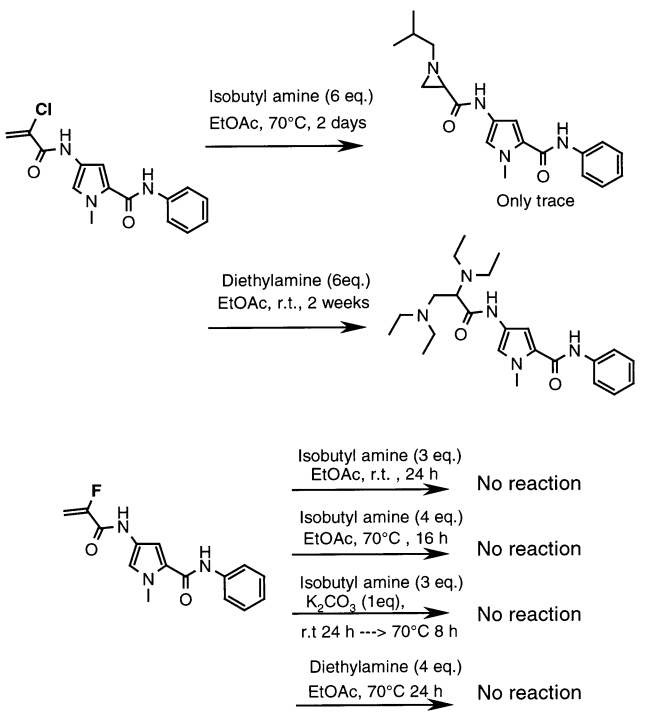
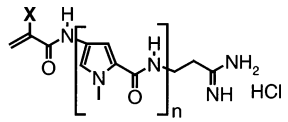


Fig. 8. Reactivity of α-chloro and α-fluoro acrylamido moieties (α-chloroacrylamino and α-fluoroacrylamino-pyrrolecarboxyanilide).

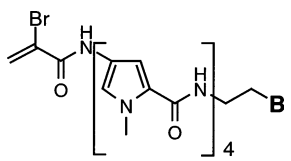
Table 2
The effect of the length of distamycin-like frame on in vitro and in vivo activity (L1210 leukemia) [a] [30]



X	N	In vitro IC_{50} nM	In vivo	
			OD mg/kg	T/C %
Br	5	23.0 ± 5.0	0.78	167
Br	4	6.3 ± 1.3	1.56	200
Br	3	98.8 ± 24.2	12.5	100
Br	2	>1300	nd	nd
Br	1	>13000	nd	nd
Cl	4	3.8 ± 1.4	1.56	133
Cl	3	96.8 ± 24.2	12.5	117

[a] IC_{50} = 50% inhibitory concentration as the mean ± SE from dose–response curves of at least two experiments; drug sensitivity determined after 4 h of continuous exposure against L1210 cells, after 48 h for other compounds; for in vivo studies cells were injected i.v. and mice were treated i.v. the day after tumor injection; OD optimal (non-toxic) dose $<LD_{10}$; T/C median survival time of treated versus untreated mice ×100.

Table 3
In vitro and in vivo activity (L1210 leukemia) of α-bromoacrylic distamycin-like derivatives modified at the amidino moiety [a] [30]



B	In vitro IC_{50} nM	In vivo	
		OD	T/C %
$C(NH)NH_2 \cdot HCl$	6.3 ± 1.3	1.56	200
$C(NCH_3)NH_2 \cdot HCl$	2.6 ± 0.7	3.13	150
$C(NCH_3)NCH_3 \cdot HCl$	1.8 ± 0.1	1.56	258
$CNHN(CH_3)_2 \cdot HCl$	1.4 ± 0.8	nd	nd
C-imidazolin-2-yl·HCl	2.4 ± 0.5	nd	nd
$NHC(NH)NH_2 \cdot HCl$	1.8 ± 0.1	1.56	196
$C(NOH)NH_2$	8.5 ± 1.5	6.25	186
$C(NCN)NH_2$	4.1 ± 0.2	3.13	157
$CONH_2$	9.0 ± 0.4	6.25	169
CN	18.1 ± 2.2	12.5	157

[a] IC_{50} = 50% inhibitory concentration as the mean ± SE from dose–response curves of at least two experiments; drug sensitivity determined after 48 h of continuous exposure against L1210 cells; for in vivo studies cells were injected i.v. and mice were treated i.v. the day after tumor injection; OD optimal (non-toxic) dose $<LD_{10}$; T/C median survival time of treated versus untreated mice ×100.

further reaction of the no longer vinylic bromide, leading to alkylation of DNA nucleophilic functions. Thus PNU-166196 might alkylate DNA only in the presence of GSH, which is the most abundant intracellular thiol, present in the millimolar range in mammalian cells [39].

In order to verify the hypothesized role of GSH, experiments of interaction of PNU-166196 and parent PNU-151807 with plasmidic DNA were performed, in the presence or in the absence of GSH. Agarose gel electrophoresis showed that both compounds induced the change of plasmid DNA, from the supercoiled form to the circular form (nicking), only in the presence of GSH, while in the absence no change occurred in the plasmid topology, at variance with TAM. The inactive fluoroacrylic analog of PNU-166196 was unable to relax plasmidic supercoiled DNA in the presence of GSH, Fig. 11.

Moreover the role of GSH on the cytotoxicity of PNU-166196 was investigated by testing the compound against tumor cells characterized by high levels of GSH, such as melphalan (L-PAM) resistant leukemia tumor cells (L1210/L-PAM), which present a three fold increase of GSH in comparison to wild L1210 cells. The cytotoxicity of PNU-166196 and parent PNU-151807 against L1210/L-PAM cells showed a three fold increase in comparison to wild L1210 cells, while the cytotoxicity of TAM was comparable on the two cell lines, Table 5.

The role of GSH on PNU-166196 cytotoxicity was investigated also by inhibiting GSH formation with buthionine sulphoximine (BSO), an inhibitor of γ-glutamylcysteine synthase. BSO was able to decrease significantly both cytotoxic and apoptotic effects of

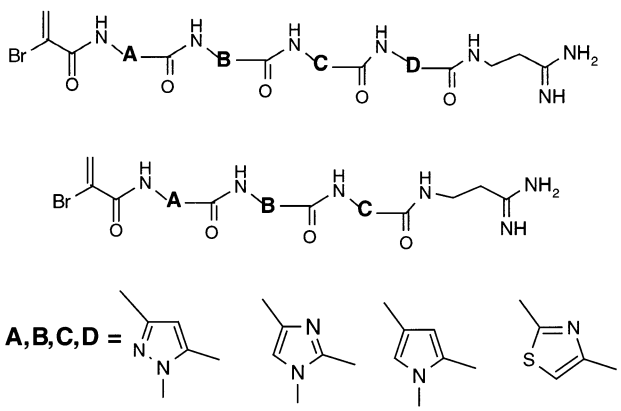


Fig. 9. Representative examples of isosteric α-bromoacrylic analogs.

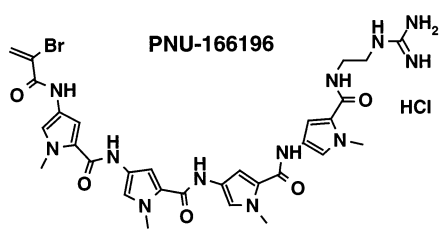


Fig. 10. PNU-166196 is undergoing phase I studies.

Table 4
PNU-166196 cytotoxicity/myelotoxicity ratio. The in vitro myelotoxicity of PNU-166196 is radically reduced compared to other minor groove binders, in particular for human hematopoietic progenitor cells

Comp.	Myelotoxicity human CFU-GM [a] IC_{50} ng/ml	Cytotoxicity tumor cells [b] IC_{50} ng/ml	In vitro cytotoxicity/myelotoxicity ratio
PNU-166196	2377	29.3	81.1
Tallimustine	290	330	0.9
Carzelesin	0.15	0.04	3.7
Bizelesin	0.18	0.17	1
Adozelesin	0.17	0.04	4.2

[a] Human colony-forming units, granulocytes-monocytes hematopoietic progenitor cells.
[b] Mean IC_{50} values of ten tumor cell lines.

PNU-166196: no relaxation
PNU-151807: no relaxation

Tallimustine : relaxation
Distamycin : no relaxation

PNU-166196 + GSH : relaxation
PNU-151807 + GSH : relaxation

α-fluoroacrylic analog of PNU-166196 + GSH : no relaxation

Fig. 11. PNU-166196 interaction with plasmidic DNA (agarose gel electrophoresis after incubation with plasmid containing sequences T4GA). Relaxation of the plasmidic DNA from the supercoiled form to the circular form.

Table 5
Cytotoxicity of PNU-166196, PNU-151807 and tallimustine versus L1210 and L1210/L-PAM leukemia cells: L1210/L-PAM murine leukemia cells present a three-fold increase of GSH levels

Comp.	IC_{50} ng/ml (48 h treatment)	
	L1210	L1210/L-PAM
PNU-166196	1.62	0.49
PNU-151807	0.86	0.26
Tallimustine	22.5	27.4

PNU-166196 on A2780 human ovarian carcinoma cells, Table 6. These findings suggest that GSH affects both the mechanism of PNU-166196 DNA interaction and cytotoxicity, with a potential value in cancer treatment.

In fact high levels of GSH and of glutathione *S*-tranferases (GSTs), the enzymes which catalyze the nucleophilic GSH reactivity [40], have been reported to play a role in the resistance of tumor cells to different anticancer drugs, such as classical mustards and *cis*-platinum [41,42]. Moreover there is evidence that a number of human tumors display increased levels of GSH and GSTπ isozyme with respect to normal tissues [43].

Table 6
Cytotoxicity and apoptotic effect of PNU-166196 on A2780 cells and A2780 cells pretreated with BSO

Treatment	Dose	Growth inhibition (%)	Apoptosis (%)
PNU-166196	1 μg/ml	50	65
	3 μg/ml	71	71
PNU-166196+	1 μg/ml	22	8
BSO 0.1 mM	3 μg/ml	31	17
BSO	0.1 mM	5	0

4. Conclusions

In conclusion these studies led to the identifcation of a new class of cytotoxic minor groove binders, α-bromoacrylamido distamycin derivatives, endowed with potent antitumor activity and reduced myelotoxicity. Compounds of this class interact with TA-rich sequences of DNA, but at variance with TAM or CC-1065-derived agents, appear unreactive in classical in vitro DNA alkylation assays. However there is evidence that these compounds may be activated in vivo by GSH, leading to irreversible DNA interaction.

PNU-166196 shows potent antitumor activity and very favorable cytotoxicity/myelotoxicity ratio and, due to its interaction with GSH, may be hypothesized to have a specific role for the treatment of tumors characterized by constitutive or therapy-induced overexpression of GSH-GST levels.

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