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The discovery of a new potential anticancer drug: a case history

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Abstract

DNA minor groove binders (MGB) represent a class of anticancer agents whose DNA sequence specificity was hypothesized to lead to high selectivity of action. Tallimustine (TAM), a benzoyl nitrogen mustard derivative of distamycin A (DST), showed excellent antitumor activity in preclinical tests, but also a severe myelotoxicity. Novel nitrogen mustard, nitrogen half-mustard and sulfur mustard derivatives of DST showing excellent activity were recently identified and SAR reported. In particular nitrogen half-mustard and sulfur mustard derivatives, as one-arm alkylating agents, represent interesting structural novelties. A further new class of cytotoxic anticancer agents is that of α-halogenoacrylamido derivatives of DST-like oligopeptides, which show an activity profile substantially improved in comparison to TAM. In particular brostallicin (PNU-166196), α-bromo-acrylamido tetra-pyrrole derivative ending with a guanidino moiety, showed high cytotoxic potency and myelotoxicity dramatically reduced in comparison to TAM and other MGB. Brostallicin binds to the minor groove but appears unreactive in classical in vitro DNA alkylation assays. About the apparent lack of DNA alkylation we speculated that an intracellular nucleophile, e.g. glutathione (GSH), could activate the reactivity of the compound leading to alkylation of DNA in vivo. Evidence of both covalent interaction of brostallicin with plasmidic DNA in the presence of GSH and of enhanced cytotoxicity in cancer cells characterized by high levels of GSH were obtained. Brostallicin was selected for clinical development and is now undergoing Phase II studies.

Keywords: Minor groove; Distamycin; Brostallicin; α-Halogenoacrylamides; Glutathione

1. Introduction

Cancer is an heterogeneous group of diseases, characterized by uncontrolled growth of the malignant cell population. The estimated worldwide incidence of different types of cancers is around 10 millions, roughly half of which in developed countries. The incidence, prevalence and mortality in the USA and in Italy are, respectively, about 1.2, 8 millions, 550 thousands and about 250 thousands, 1 million, 80 thousands [1–3].

In spite of impressive progress in diagnosis, surgery and therapy, occurred since the Sixties, the overall cancer mortality, even somewhat declined in late Nineties in some countries, is still very high, with some exceptions for some specific tumor types. For instance in the USA the cancer deaths 2000 versus 1985 in thousands were, respectively, 160 versus 120 for lung; 110 versus 60 for colon-rectum; 41 versus 38 for breast; 32 versus for prostate; 24 versus 19 for urinary tract; 14 versus 12 for ovary [1,4]. The classical 5-year survival rate for the average of the more common cancers is about 60%, but is as low as 14% for lung cancer. In general the 5-year survival rate is increasing, however, this reflects more the great progress in early diagnosis, as the large scale practices of mammography, PSA and Pap test, than the real capability of therapeutic control [5].

A dramatic decrease of mortality occurred, at least in developed countries, only for those cancers in which both early diagnosis and full organ surgery are possible, as in the case of uterus and cervix or for which a substantial drop of incidence occurred, as in the case of the stomach. In the USA, the cancer mortality 1995 versus 1950 dropped by 67% for uterus, 76% for cervix, 80% for stomach, while e.g. both mortality and incidence increased by about 250% for lung cancer [5].

In spite of the large number of available chemotherapeutic agents the medical need is still largely unmet. The main reasons are: the lack of selectivity of conven-

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tional drugs, leading to toxicity; the metastatic spreading, implying early tumor implantation in organs other than primary site; the heterogeneity of the disease, comprising about 100 types of cancer; the intrinsic or acquired resistance to chemotherapy developed after few therapeutic cycles (multi-drug resistance).

A number of innovative strategies are in development, aimed to target the malignant abnormalities of tumor cells arising from the activation of tumor promoter genes or inhibition of tumor suppressor genes. Some focused molecular targets are e.g. oncogene encoded proteins acting as signal transducer like RAS, cell cycle targets as cyclin-dependent kinases, telomerase. In alternative, angiogenesis inhibition may prevent the formation of tumor blood vessels, thus limiting the tumor ability to grow and metastasize. Focused molecular targets of angiogenesis cascade are e.g. the proteases degrading cellular matrix, endothelial tyrosine kinases, $\alpha v\beta 5$ transmembrane integrins. However, it will likely take some years to fully define the role of innovative agents in preventing disease progression. Therefore, cytotoxic drugs will continue to represent a chief part of the therapy in the near future, and this implies a need for new agents with better activity and safety profile.

2. Distamycin-derived nitrogen mustards

The putative mode of action of many antitumor agents involves a DNA damage, either by direct binding of the drug to DNA or to DNA-binding proteins such as topoisomerases. Most of DNA-interacting agents have only a limited degree of sequence specificity, which imply that they may hit most cellular genes. The minor groove represents a vulnerable site of attack normally unoccupied, while most of DNA interactive proteins bind to the major groove. However, the minor groove is the site of non-covalent, reversible, highly specific interaction with thymine-adenine (TA)-rich sequences for several molecules of MW less than 1 kDa endowed with antibacterial and antiviral activities. One of these molecules is distamycin A (DST) [6], which show a highly selective reversible interaction with TA sequences containing at least four T4GA base pairs [7].

DNA minor groove binders (MGB) able to damage irreversibly DNA, e.g. to alkylate DNA, may represent a new class of cytotoxics, whose increased gene specificity may provide significant improvement in potency and selectivity [8]. The main representatives of this class that were clinically tested in the recent past are tallimustine (TAM) [9], a benzoic acid nitrogen mustard derivative of DST, Fig. 1, and the compounds structurally derived from antibiotic CC-1065, i.e. adozelesin, carzelesin, bizelesin [10].

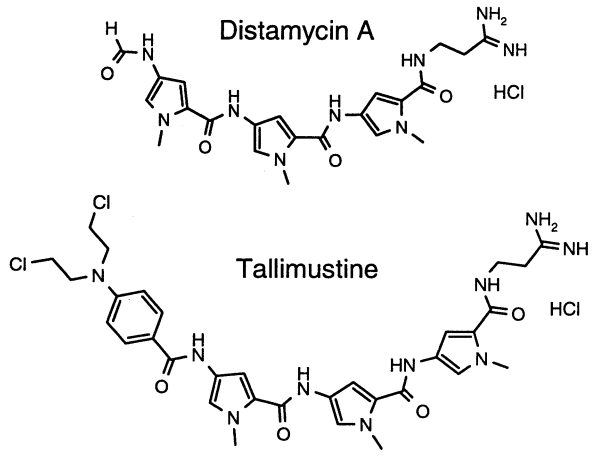
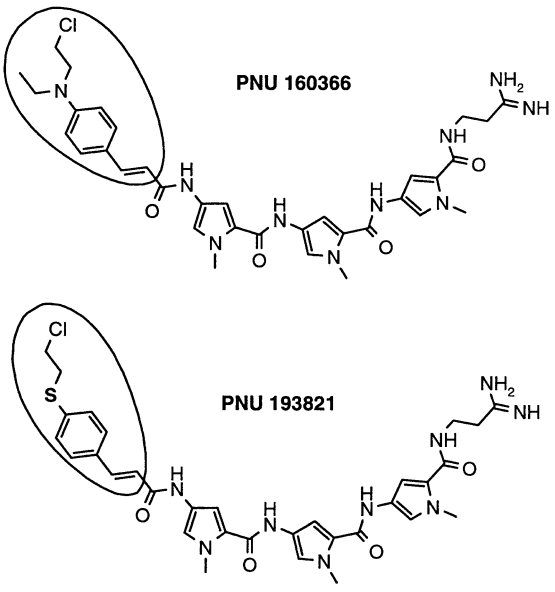


Fig. 1. Distamycin A and its benzoyl nitrogen mustard tallimustine. IC_{50} against L1210 murine leukemia. Distamycin A: 10.0 μM, Tallimustine: $IC_{50} = 68.5$ nM.

TAM showed cytotoxicity against L1210 murine leukemia more than two orders of magnitude higher then DST and more than one order of magnitude higher then classical nitrogen mustard melphalan [11]. It may be hypothesized that distamycin-driven DNA binding increases the TAM concentration near the DNA target, avoiding as much as possible a specific alkylation of biological nucleophiles, as e.g. glutathione (GSH), which is a major drawback of classical mustards [12]. TAM showed excellent antitumor activity in the preclinical tests, however, its clinical evaluation showed a severe myelotoxicity [13] and its development was discontinued. Nevertheless TAM has represented an important model for the design of new minor groove alkylating agents having proved the possibility of obtaining potent antitumor activity by tethering a moiety of mild chemical reactivity, such as that represented by benzoic acid nitrogen mustard, to a DNA selective binding-frame, thus leading to DNA alkylation with highT4GA sequence specificity.

Derivatives of DST called a particular interest also 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 units [14–20]. Moreover the role of the chemical reactivity of the phenyl mustard and that of basicity of the amidino moiety were specifically investigated by us [21,22]. The medicinal chemistry and the SAR of distamycin-derived nitrogen mustards, has been reviewed by several authors including us [23,24] and will not be further discussed here.

However, a specific finding arising from our studies seems worthwhile of a particular mention: the potent activity of one-arm mustards derived form DST. This is well exemplified by two cinnamic derivatives, the nitrogen ethyl half-mustard PNU-160366 and the sulfur mustard PNU-193821 [21,25]. Fig. 2. Both compounds



IC_{50} against L1210 murine leukemia

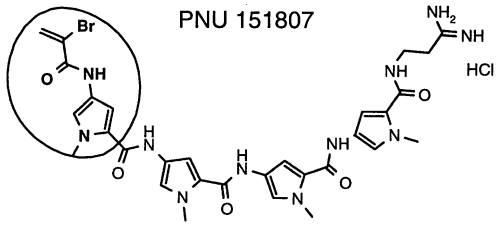
PNU 160266: 4.0 nM - PNU 193821: 0.9 nM
Tallimustine: 68.5 nM

Fig. 2. Cinnamic nitrogen half-mustard and sulfur mustard derivatives of distamycin. IC_{50} against L1210 murine leukemia.

show the possibility of achieving potent activity by tethering a reactive one-arm mustard to a minor groove binding ligand, while classical nitrogen half-mustards and sulfur mustards are substantially non cytotoxic [26], due to the impossibility of crosslinking the DNA strands [27]. Sulfur mustard PNU-193821 was the most potent DST-derived cytotoxic found in our laboratories (IC_{50} 0.9 nM against L1210 leukemia). The corresponding sulfoxide derivative shows a substantial lack of cytotoxicity, suggesting that the formation of a thiiranium cation, which is no more possible in the case of the sulfoxide, may play a key role for the mechanism leading to cytotoxicity.

3. Distamycin-derived α-halogenoacrylamides

A new class of cytotoxic MGB showing α-bromo or chloro-acrylamido moiety linked to DST or DST-like frames was identified in our laboratory [11]. The first lead of this class, PNU-151807, Fig. 3, the α-bromoacrylamido derivative of four-pyrrole DST homologue, showed high cytotoxicity and in vivo activity. Moreover PNU-151807 was found to bind to DNA minor groove but unable to alkylate minor groove AT-rich sequences in classical in vitro experiments [28] at variance with TAM.



IC_{50} against L1210 murine leukemia

PNU-151807: 6.3 nM - Tallimustine: 68.5 nM

Fig. 3. PNU-151807: the lead of distamycin-derived α-halogenoacrylamides. IC_{50} against L1210 murine leukemia.

The cytotoxicity of the compounds of this series depends upon the chemical reactivity of α-halogenoacrylic moiety, that we studied with simple model compounds, i.e. 4-(α-haloacrylamido)-pyrrole-2-carboxyanilides. The structures of derivatives obtained by the reaction of these compounds with amines or the imidazole ring are in agreement with a first-step Michael nucleophilic attack, followed by a further reaction leading to a second nucleophilic substitution or to β-elimination [24] Fig. 4.

The dramatically different reactivity toward nucleophilic attack of α-bromoacrylamido and of α-fluoroacrylamido moieties corresponds to the dramatically different cytotoxicity of α-bromo and α-fluoro-acrylamido DST derivatives, the former very potent, the latter inactive. Also acrylamido DST derivative is devoid of activity confirming the requirement of a reactive halogen [29].

The relevant activity of PNU-151807, its cytotoxicity/myelotoxicity ratio significantly better than that of TAM [30] and its unusual mechanistic features, prompted us to the synthesis of new halogenoacrylic derivatives of DST and congeners. 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 rings.

The three pyrrole unit DST derivatives, both bromo and chloro, are about one order of magnitude less cytotoxic than the corresponding four pyrrole congeners. The same decrease of activity occurs with α-bromo derivatives with two and one-pyrrole units, which are, therefore, devoid of significant activity (Table 1). This feature was classically considered to arise from a tighter DNA binding depending on the increased multiplicity of interaction between the pyrrolecarboxamide units and minor groove TA-rich sequences [31]. However, recent permeability data obtained using a Caco-2 cells line (Table 2) show that in the series of α-bromoacrylic polypyrrole derivatives there is a progressive strong increase of permeability from one to four pyrrole compounds, which goes together with their increased

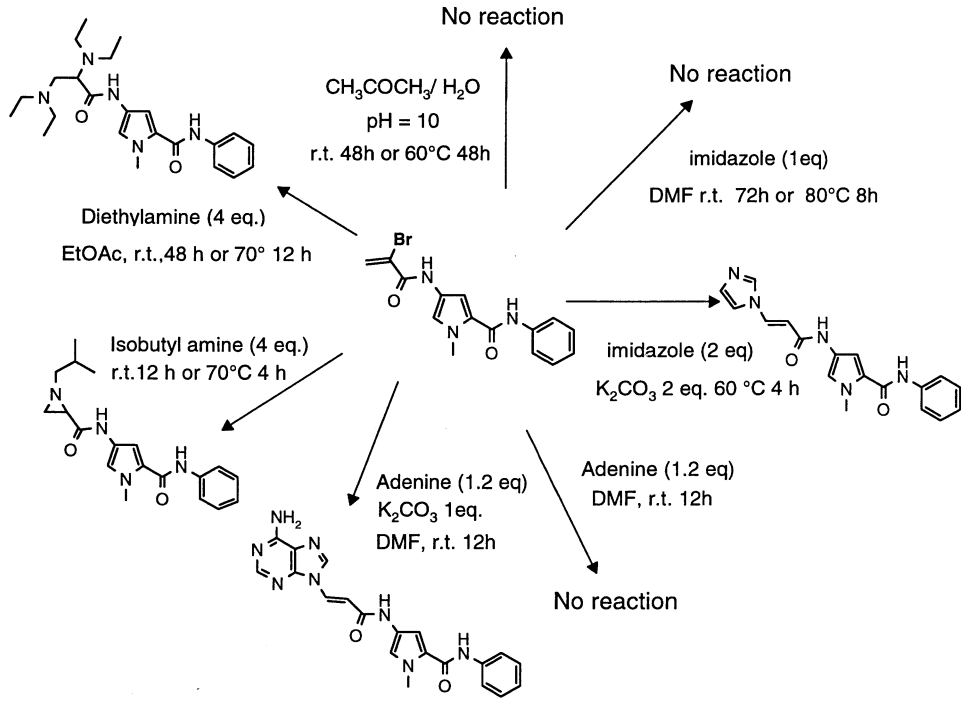
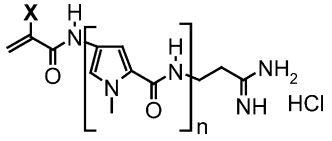


Fig. 4. Reactivity of α-bromoacrylamido moiety (α-bromoacrylamino-pyrrolecarboxyanilide). α-Fluoroacrylamino-pyrrolecarboxyanilide does not react in the same or harder conditions.

Table 1
The effect of the length of distamycin-like frame on in vitro and in vivo activity (L1210 leukemia)

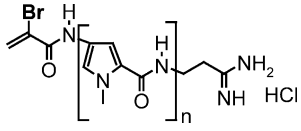


X	*n*	In vitro IC_{50} (nM)	In vivo OD (mg/kg)	*T/C* (%)
Br	5	23.0 [a] ± 5.0	0.78	167
Br	4	6.3 ± 1.3	1.56	200
Br	3	98.8 ± 24.2	12.5	100
Br	2	> 1300	n.d.	n.d.
Br	1	> 3000	n.d.	n.d.
Cl	4	3.8 ± 1.4	1.56	133
Cl	3	96.8 ± 24.2	12.5	117

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

[a] 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 tumorinjection; O.D., optimal (non toxic) dose < LD10; T/C = median survival time of treated versus untreated mice × 100.

Table 2
Caco-2 cells permeability for a series of α-bromoacrylamido derivatives



n	Papp × 10^{-6} (cm/s) [a]	ClogD, pH 7.4 [b]	IC_{50} (nM)
4	38.20	−6.63	6.3
3	15.21	−6.0	98.8
2	5.58	−5.38	1300
1	0.69	−4.75	> 3000

[a] Apparent permeability, method of P. Artusson.
[b] ACD calculated LogD at pH 7.4.

cytotoxicity. This may represent an additional explanation for high cytotoxicity of derivatives with three or more pyrrole units and suggests the existence of an active transport mechanism for these compounds, whose permeability cannot by related to a passive transport, due to high MW, full ionization, high hydrophilicity and high hydrogen bonding capability.

As far as the role of strongly basic amidino moiety, typical of DST, is concerned, the activity of the parent amidino lead PNU-151807 is fully maintained not only by basic amidino-like compounds, but also by non-basic

amidino derivatives such as amidoxime and cyanoamidine and by carboxyamide derivative [32]. The amidino moiety, due to its strong basic nature, implies the total protonation at any biological condition, which may play a key role for DNA binding. 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.

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. Some of these derivatives appear significantly more potent than TAM, however, even best compounds of this series do not show a profile of activity better than that of the corresponding pyrrole analogs.

Extensive antitumor investigation among the whole series of oligopeptidic α-bromoacrylic derivatives led to the selection for the clinical development of PNU-166196, INN brostallicin, an α-bromoacrylamido-tetrapyrrole derivative ending with a guanidino moiety (Fig. 5), which is presently undergoing Phase II clinical trials.

Brostallicin shows a broad spectrum of activity, circumvents resistance to alkylating agents and camptothecins, results more active than TAM in inducing apoptosis and shows an outstanding favorable cytotoxicity/myelotoxicity ratio. In fact its mean IC_{50} against a series of tumor cell lines is about eighty times lower than its IC_{50} on human CFU-GM hematopoietic progenitors cells [33]. The dramatic improvement of the cytotoxicity/myelotoxicity ratio of brostallicin in comparison with TAM and other MGB represents a determinant feature to make possible the reaching of effective therapeutic doses that was not possible in the case of TAM.

This compound, as the parent PNU-151807, appears unreactive in DNA in vitro alkylation assays [33]. About 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 further reaction, leading to alkylation of DNA nucleophilic functions. Fig. 6. Thus brostallicin might alkylate DNA only in the

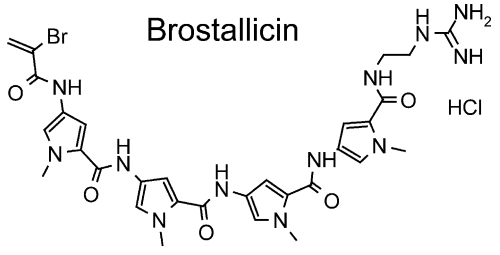


Fig. 5. Brostallicin (PNU-166196) undergoing Phase II studies.

presence of GSH, which is the most abundant intracellular thiol, present in the millimolar range in mammalian cells [34].

Our hypothesis was supported by experiments of interaction of brostallicin and parent PNU-151807 with plasmidic DNA, 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. Inactive fluoroacrylic analog of brostallicin was unable to relax plasmidic supercoiled DNA in the presence of GSH [35].

Moreover the cytotoxicity of brostallicin and parent PNU-151807, against melphalan-resistant leukemia cells, characterized by high levels of GSH, showed a three-fold increase in comparison to wild L1210 cells, while the cytotoxicity of TAM was comparable on the two cell lines. The role of GSH on brostallicin cytotoxicity was further confirmed by the fact that inhibition of GSH formation, with buthionine sulfoximine (BSO), led to a significant decrease of both cytotoxic and apoptotic effects on A2780 human ovarian carcinoma cells [35]. Evidence for the role of GSH on brostallicin efficacy has been obtained also in vivo. Clones expressing high levels of GSH-S-transferase-π (GST-π), obtained from A2780 cell line after transfection with human GST-π gene, showed a three-fold increased sensitivity to brostallicin in comparison to transfected ones with low GST-π expression. In tumors developed in nude mice after implantation of GST-π-transfected clones brostallicin showed greater antitumor activity. Finally recent experiments proved that while the reaction of brostallicin and GSH alone is negligible within few minutes, the presence of GSTs, particularly of GST-π significantly enhance the reaction [36]. These findings suggest that GSH may affect both the mechanism of brostallicin–DNA interaction and antitumor activity, with a potential value in cancer treatment. In fact high levels of GSH and of GSTs, the enzymes which catalyze the nucleophilic GSH reactivity [37], have been reported to play a role in the resistance of tumor cells to different anticancer drugs, such as classical mustards and *cis*-platinum [38]. There is the evidence moreover that a number of human tumors display increased levels of GSH and GST-π isozyme in respect to normal tissues [39].

4. The synthesis of brostallicin and congeners

The syntheses of nitrogen mustards, sulfur mustards and α-halogenoacrylic DST and DST-like derivatives cited in this paper were already reported [11,21,22,25,31]. However, some synthetic pathways are outlined as representative examples of the syntheses

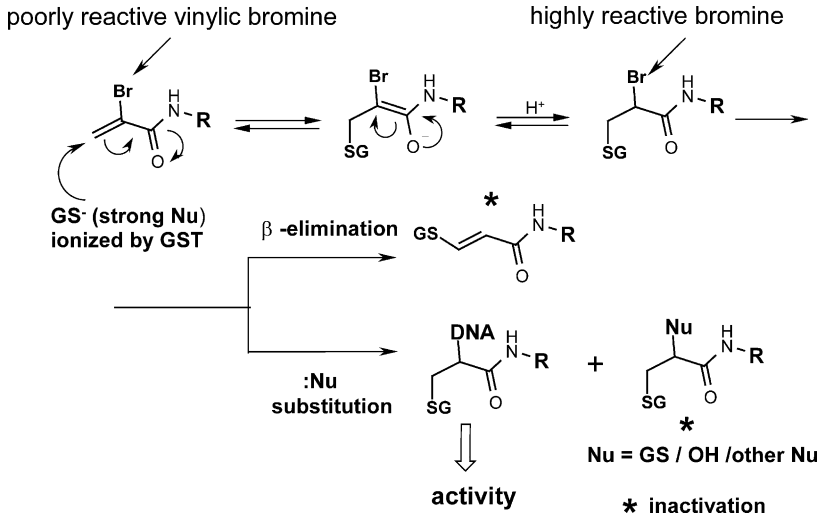


Fig. 6. Putative role of GSH in the interaction of α-bromoacrylic derivatives and DNA.

of DST derivatives. In the case of tri-pyrrole mustards, which represent the majority of tested mustards, the synthesis was based upon the coupling of the appropriate mustard acid with desformyldistamycin, in turn easily obtained by acidic hydrolysis of distamycin A. Most of the mustard derivatives modified at the amidino group were obtained by the direct reaction of the parent amidine mustard with the appropriate amine derivative, Fig. 7. The occurrence of this reaction on amidine moiety, in good yields, appears noteworthy and underlines the mild electrophilic reactivity of our nitrogen mustard moieties.

In the case of tetra-pyrrole α-halogenoacrylic derivatives, which represent the majority of α-halogenoacrylic tested compounds, the synthesis was based upon the coupling of the appropriate 4-(α-haloacrylamido)-pyrrole-2-carboxylic acid with desformyldistamycin or amidino-modified *N*-desformyldistamycin analogs, Fig. 8. The latter were obtained in turn by the direct reaction of the distamycin A with the appropriate amine derivative and subsequent hydrolysis. Fig. 7. This pathway was applied also to the synthesis of analogs in which the pyrrole ring near the α-halogenoacrylic moiety was replaced by other azoles. The original synthesis of brostallicin required, however, an intermediate desformyldistamycin analog bearing a guanidino moiety which was obtained by a step-by-step total synthesis from guanidinoethylamine, by iterative acylation with 4-nitro-pyrrolecarboxylic acid chloride and catalytic reduction of the nitro group Fig. 9.

5. Conclusions

Our studies led to the identification of a new class of cytotoxic MGB, α-bromoacrylamido DST-like derivatives, endowed with potent antitumor activity and reduced myelotoxicity. Compounds of this class interact

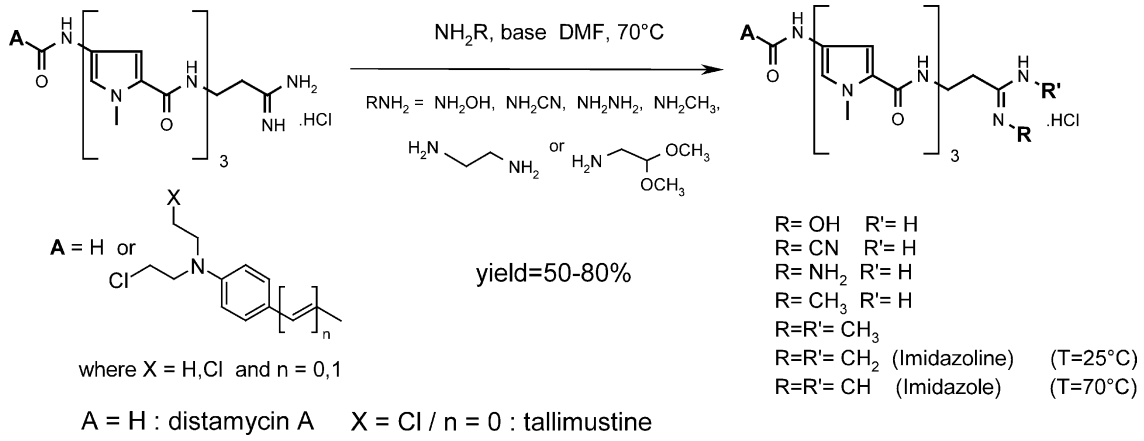


Fig. 7. A general method of synthesis of distamycin and tallimustine analogs modified at the amidino group. Replacement of the amidine by: amidoxime, amidrazone, cyanamidine, methylamidines, imidazoline, imidazole.

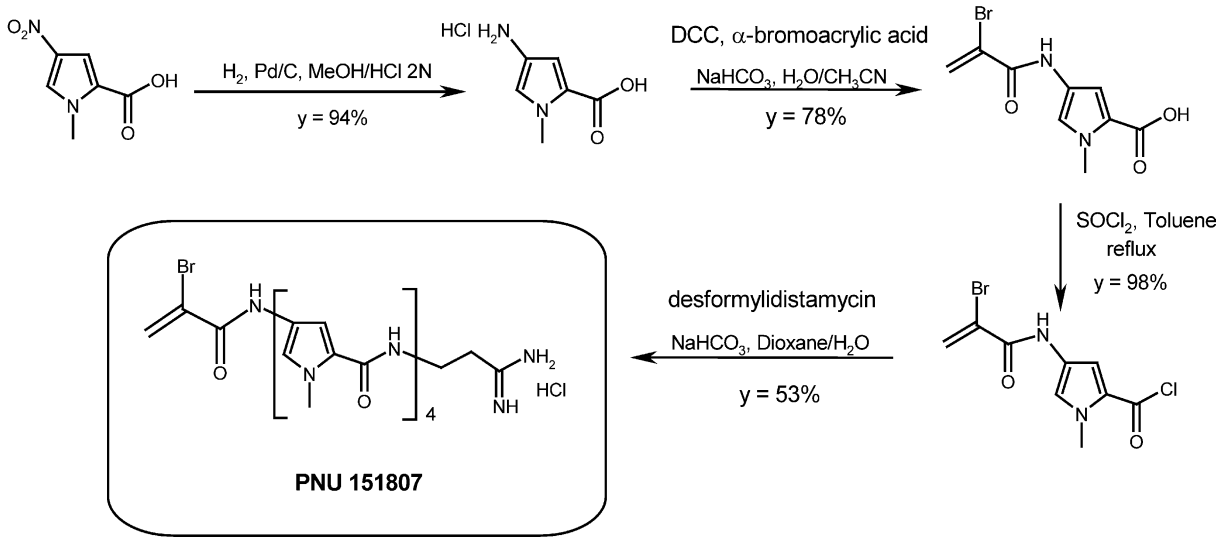


Fig. 8. Synthesis of PNU 151807 as representative example of α-halogenoacrylic derivatives.

with TA-rich sequence of DNA, but at variance with other MGB, appear unreactive in classical in vitro DNA alkylation assays. There are evidences, however, that these compounds may be activated in vivo by GSH, leading to irreversible DNA interaction. Brostallicin (PNU-166196), now undergoing Phase II clinical trials, shows potent antitumor activity, very favorable cytotoxicity/myelotoxicity ratio, is well tolerated in patients (Phase I) and, due to its interaction with GSH, may have a specific role for the treatment of tumors characterized by constitutive or therapy-induced overexpression of GSH-GST levels.

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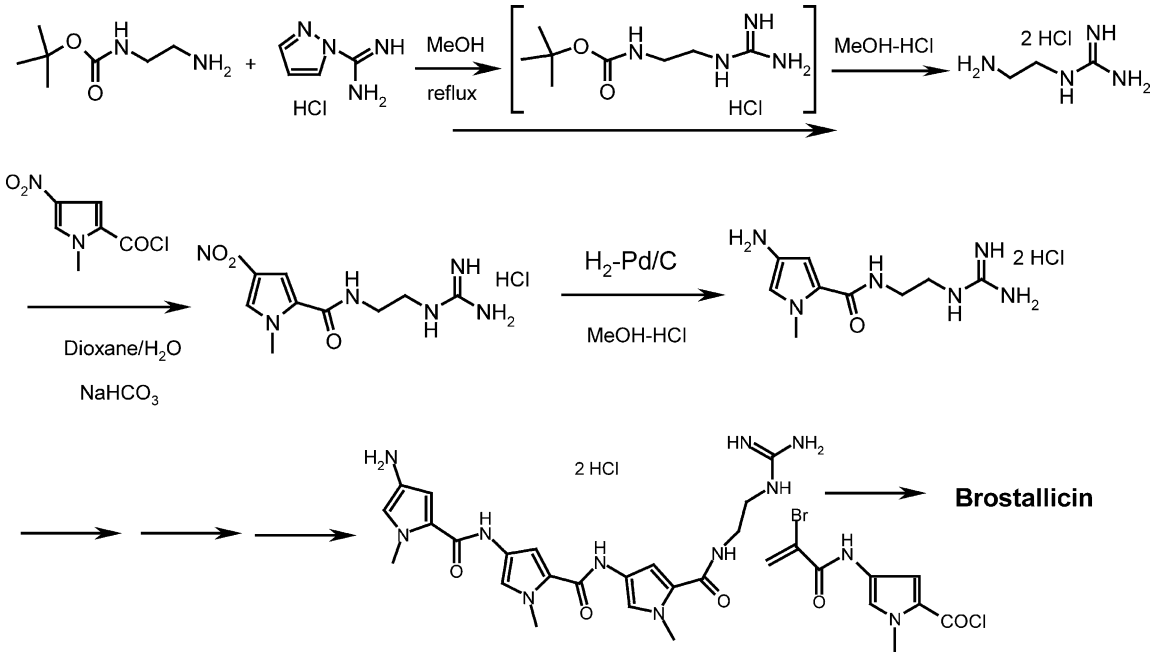

Fig. 9. Synthesis of brostallicin.

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