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Review Article

DNA minor groove binding agents

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CONTENTS

Abstract

Minor groove binding agents are molecules able to interact with the minor groove of DNA and to covalently bind residues lying in this groove. A common characteristic of this class of anticancer agents is their peculiar mechanism of action. In fact, the majority of them have a mechanism of action very distinct from that of the classic major groove DNA interacting agents and more in common with that of classic anticancer agents used in the clinic. Minor groove binding agents are characterized by potent antitumor activity *in vitro* and *in vivo*. One of the major limitations in their clinical use has been the potent and often unexpected myelotoxicity which prevents using potentially active doses. Many efforts have been made in the past to synthesize new minor groove binding agents retaining high antitumor activity and an acceptable toxicity profile. Molecules such as brostallicin and trabectedin represent clear examples of minor groove binding agents with relatively moderate undesired effects which are now in clinical evaluation. The peculiar mechanism of interaction of brostallicin with glutathione and glutathione-*S*-transferase, and the peculiar interaction of trabectedin with the nucleotide excision repair system, make these molecules particularly attractive for combination studies with classic anticancer agents with which synergism has been described at preclinical levels. Based on the interesting features of these new molecules, studies on minor groove DNA binding agents are still warranted and either natural or synthetic compounds are continuously being evaluated for their antitumor properties.

Introduction

A large number of molecules used in cancer chemotherapy interact with DNA, the majority of which interact with DNA bases located in the major groove of DNA since in this region there are many nucleophilic sites which are hit by electrophiles.

The minor groove of DNA is relatively less accessible than the major groove and has much fewer nucleophilic sites to be hit. Nevertheless, minor groove binders (MGBs) represent a very attractive class of compounds for their unique mechanism of action and potent antitumor activity. From a mechanistic point of view, the MGBs are almost invariably characterized by a very high DNA sequence specificity, higher than that observed with major groove binders (1, 2).

MGBs are characterized by very potent *in vitro* cytotoxicity and by high *in vivo* antitumor activity in experimental systems. Initial clinical evaluation of MGBs revealed, however, a very high toxicity, mainly myelotoxicity, which raised questions about the clinical usefulness of this class of compounds. The development of novel second- and third-generation minor groove binders, along with the development of new natural minor groove binders, partially solved this problem, rendering these molecules more manageable. In this review we will analyze some of the molecules with the most interesting features, focusing on a few as prototypes of the entire class and their clinical status.

Distamycin-like molecules

Distamycin A is an antiviral compound structurally able to noncovalently bind DNA in the minor groove. The compound itself does not possess activity against cancer cells either *in vitro* or *in vivo* (2). However, starting from its structure, a number of compounds have been synthesized with the aim of producing molecules able to covalently interact with DNA in the minor groove. The rationale behind this structure-related design was the possibility to drive molecules to the minor groove through the distamycin moiety and to render this binding tighter by adding a substituent able to alkylate adenines or thymine (2-4).

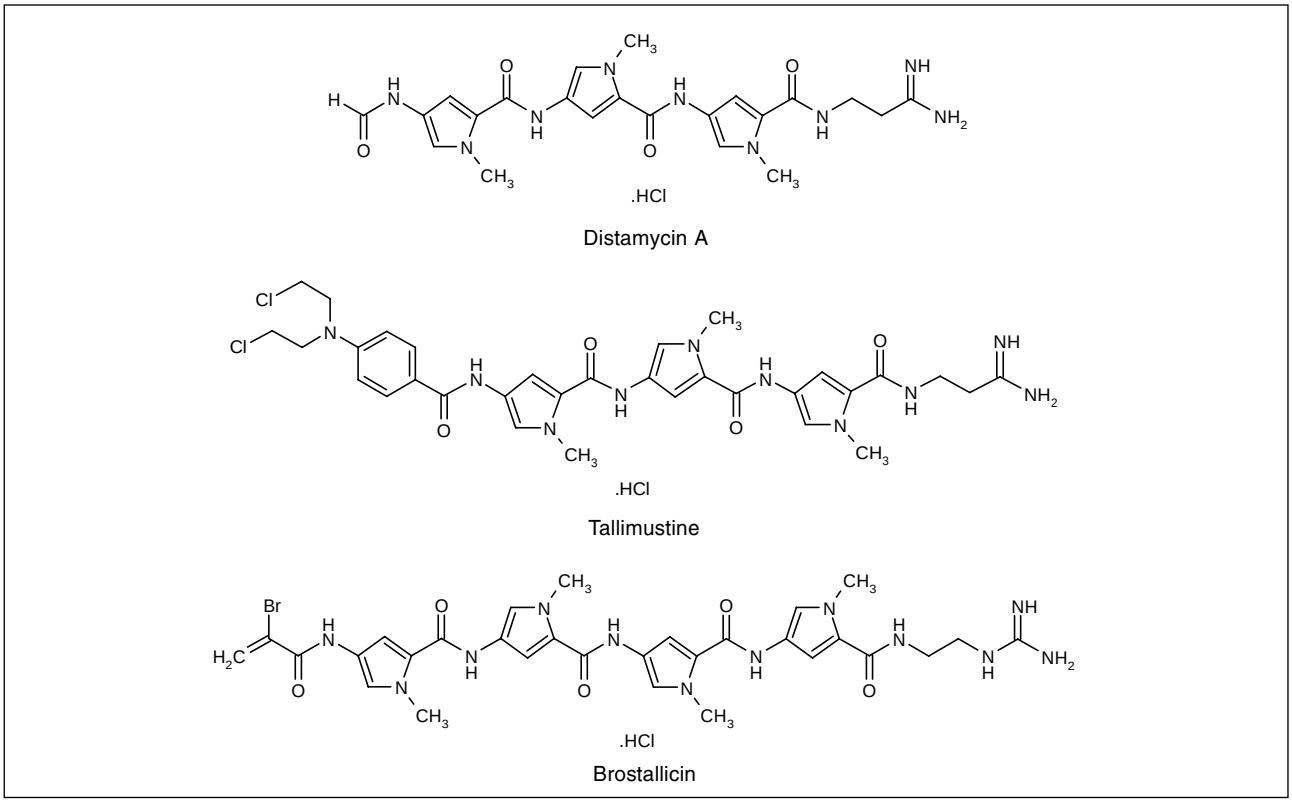

Fig. 1. Chemical structures of distamycin A, tallimustine and brostallicin.

Molecules unrelated to distamycin, such as CC-1065, aldozelesine and carzelesine, were among the first minor groove binders shown to possess high *in vitro* cytotoxicity against tumor cell lines of different origin. Although the molecules were able to covalently bind DNA in the minor groove in AT-rich regions, they were relatively unspecific in binding stretches of As or Ts with only slight preferential binding for certain sequences. Given the high potency of these molecules and the promising *in vitro* activity against cancer cell growth, a selection of new MGBs with more sequence-specific DNA binding was one of the reasons for the development of distamycin-like molecules. Attempts to combine the AT sequence specificity with an electrophilic moiety, with the aim of increasing cytotoxicity against tumor cells in comparison to that of distamycin, led to the use of the pyrrole amide framework of distamycin as a DNA binding vector tethering different electrophilic moieties (Fig. 1). Benzoic acid mustard-distamycin A conjugates with different numbers of pyrrole rings demonstrated that increasing the number of pyrrole units increased the cytotoxicity (3, 5, 6). The tripyrrole derivative was found to be the most sequence-specific of the series. The distamycin structure targets the nitrogen mustard from the major groove of DNA toward the minor groove with a DNA sequence specificity completely different from that reported for the benzoic acid mustard, which, like the classic major groove alkylating agents, alkylates DNA. Structure-activity studies clearly indicated that loss of alkylation ability was associated with a dramatic loss of *in vitro* cytotoxicity, implying that the mechanism of action of this class of compounds is related to their covalent sequence-specific DNA interactions.

Tallimustine

Tallimustine is the prototype of this class. This compound is a bis-2-chloroethylaminobenzoyl distamycin derivative, fusing in one molecule the distamycin frame and the alkylating moiety of the alkylating agent melphalan (Fig. 1). DNA footprinting experiments, together with NMR studies, showed that the pyrrole rings of the distamycin portion are located within the minor groove targeting the molecule in the AT-rich domain of B-DNA, as expected, and allowing the alkylating moiety of melphalan to bind the nucleotide residues present in the DNA minor groove (5, 7). Studies evaluating the DNA sequence specificity of tallimustine showed that this compound was extremely sequence-specific, being able to alkylate the N3 of adenine only when this was present in the sequence TTTTGA (6). This high DNA sequence specificity was associated with greater cytotoxicity than distamycin A or melphalan. Furthermore, tallimustine showed promising antitumor activity *in vivo* against murine and human tumor xenografts in mice. Interestingly, tallimustine was found to be active against tumors resistant to melphalan (8).

In vitro studies indicated that the cytotoxicity of tallimustine was not due to a different intracellular concentration since the compound had a cellular uptake and retention similar to that of distamycin.

When radiolabeled tallimustine was used, a low but measurable covalent binding to DNA was found in intact cells (5). Comparing the alkylating activity of tallimustine with that of melphalan or other nitrogen mustards, a clear weaker alkylating effect was found for tallimustine. Considering the higher *in vitro* cytotoxic potency of tallimustine, it was speculated that the relative sequence specificity, rather than the total unspecific alkylation of DNA, could be important for the antiproliferative activity of alkylating agents. Initial clinical trials with tallimustine were nonetheless disappointing due to its severe myelotoxicity, which prevented the use of doses predicted to have antitumor activity (9-11).

From tallimustine, a number of newer distamycin derivatives have been synthesized and tested *in vitro* for their antitumor activity. Based on the previous myelotoxicity data obtained in patients, the new compounds were tested not only against cancer cell lines growing *in vitro* but also against human myeloid cells (12). This new screening strategy allowed the selection of a new interesting class of compounds, the α-bromoacrylic derivatives of distamycin (13).

Brostallicin

DNA footprinting experiments have shown that α-bromoacrylic derivatives of distamycin are able to bind DNA in the minor groove (13, 14). However, all these derivatives are characterized by the fact that, in contrast to tallimustine, they are not able to covalently bind DNA *in vitro* when naked DNA is used as template (13). The first compound of the series to be studied was PNU-151807, whose activity was characterized both *in vitro* and *in vivo* (14-16). The comparative activity of this class against cancer cells and normal myeloid cells was in favor of cancer cells, while tallimustine showed similar activity in the two systems (17). New derivatives were synthesized from PNU-151807, the best candidate being brostallicin (Fig. 1) which was selected for further studies (18). Mechanistic studies of the compound revealed unique features.

Structure-activity studies showed that α-bromo- and α-chloroacrylamido derivatives possessed significant cytotoxicity, while the α-fluoroacrylamido and acrylamido derivatives were inactive. These data indicated that the reactivity of the α-halogenoacrylic moiety was a key determinant of drug-induced cytotoxicity (18).

Initially it was found that brostallicin not only was active in melphalan-resistant cells, but also displayed even higher activity compared to that obtained in parental cells (19). Since the melphalan-resistant cell line was characterized by an increased GSH content, a new mechanism of action was postulated.

In the cell and in the presence of an intracellular reactive nucleophilic species such as GSH, α-bromoacrylic derivatives would perform a first-step Michael-type attack followed by a further reaction leading to the alkylation of DNA nucleophilic sites (Fig. 2) (19, 20). As a consequence, brostallicin should alkylate DNA only in the presence of cellular thiols (particularly GSH). Furthermore, an important role in this reaction appears to be played by the glutathione-*S*-transferase (GST), particularly by the pi isoform (19, 20). This hypothesis has been confirmed experimentally.

Brostallicin (and the parent compound PNU-151807), in fact, induced the change of plasmidic DNA from supercoiled to circular form (nicking) only if GSH and GST were present in the reaction. The inactive α-fluoroacrylic analogue of brostallicin was, on the contrary, unable to relax

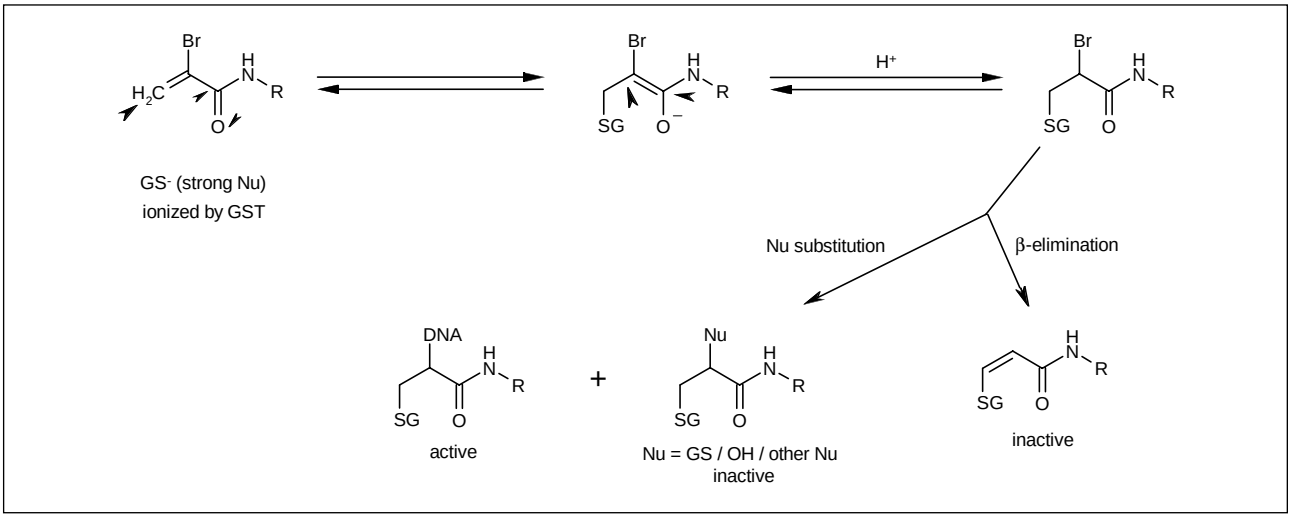

Fig. 2. Role of GSH in the interaction between α-bromoacrylic derivatives and DNA.

plasmidic supercoiled DNA even in the presence of both GST and GSH (19, 21).

The brostallicin-DNA interaction was further analyzed by Taq polymerase stop assay and compared to tallimustine in the presence or in the absence of GSH and GST. While tallimustine was able to alkylate DNA *per se*, brostallicin (as expected) was completely unable to produce any alkylation in any of the selected DNA interacting regions. However, when a GST and GSH mixture was added to the reaction, brostallicin was able to bind covalently AT-rich regions on DNA with a sequence specificity different from that previously reported for tallimustine (22). Additional evidence has been produced since then in favor of this mechanistic hypothesis: the use of glutathione-depleting agents such as BSO reduced the activity of brostallicin. Transfection of human GST-pi cDNA in cells not expressing or expressing low levels of GST-pi enhanced the activity of brostallicin *in vitro* and *in vivo* (19).

Interestingly, many human cancers express relatively high levels of GST-pi, which is often associated with resistance to classic alkylating agents such as cisplatin or melphalan. Brostallicin would therefore offer the advantage of being more active in those tumors expressing high GST-pi level. Furthermore, the combination treatment of brostallicin and cisplatin would be likely to produce synergistic effects, and preclinical experiments performed in mice strongly support this. Brostallicin was not only synergistic with cisplatin but also produced a therapeutic gain when combined with clinically used drugs such as docetaxel, irinotecan and doxorubicin (23). This therapeutic advantage was obtained without significant increase in toxicity, supporting the clinical use of brostallicin in combination regimens. A final important point to be stressed in regard to combination experiments is that drugs such as cisplatin induce a transient increase in the expression of GST-pi (23, 24), which could enhance brostallicin activity when administered following cisplatin. This hypothesis has been proven at the experimental level, as the treatment sequence of cisplatin following brostallicin was more active than the reverse scheme in human tumors transplanted in nude mice (23).

The toxicological profile of brostallicin demonstrated lower myelotoxicity than tallimustine and other minor groove binders in different animal species (12). The toxicity studies performed in monkeys and mice showed dose-dependent myelotoxicity which was reversible. When single *versus* repeated administrations were compared, the latter were better tolerated than the former (20).

Brostallicin has been used in the clinic and two phase I studies were performed to assess different treatment schedules (25, 26). For both of the studies, the drug was administered intravenously over 10 min. The maximum tolerated dose was 10 mg/m^2 for the schedule using a single administration every 3 weeks and 2.4 mg/m^2/week for the schedule in which a weekly schedule repeated 3 times was used. The dose-limiting toxicities were neutropenia and thrombocytopenia. Some nausea and vomiting were also reported. The nadir of toxicity occurred 14 days after treatment and in the majority of the patients the counts recovered by day 21. In these studies, 1 partial response was achieved in a patient with gastrointestinal stroma tumor and several disease stabilizations were obtained. The pharmacokinetic parameters obtained from these phase I studies showed a plasma half-life of approximately 5 h, moderate tissue distribution with low clearance. The drug is now in phase II clinical studies for the treatment of both hematological and solid tumors.

Marine compounds

Compounds obtained from organisms of marine origin are gaining more and more importance in the field of anticancer agents. Some of the structures evaluated for their antitumor activity are chemically very complex although DNA still remains the major target of activity for some of them. Studies on the mechanism of action of natural products of marine origin have defined interesting molecules now approaching the clinic. Among these, trabectedin is one of the most interesting compounds because of its peculiar mechanism of action and outstanding antitumor activity observed at the preclinical level.

Trabectedin

Trabectedin (ET-743) has a complex chemical structure consisting of three tetrahydroisoquinoline rings (Fig. 3) defined as subunit A, B and C. Based on the available experimental data, the cytotoxic effects of the drug are likely to depend upon its interaction with DNA. It has in fact been shown that trabectedin binds to the N2 position of guanine in the minor groove of DNA with a certain degree of sequence specificity such as 5'-PuGC and 5'-PyGG, stabilized by a hydrogen bonding network (27). NMR studies showed that subunits A and B of the molecule are responsible for DNA recognition and binding,

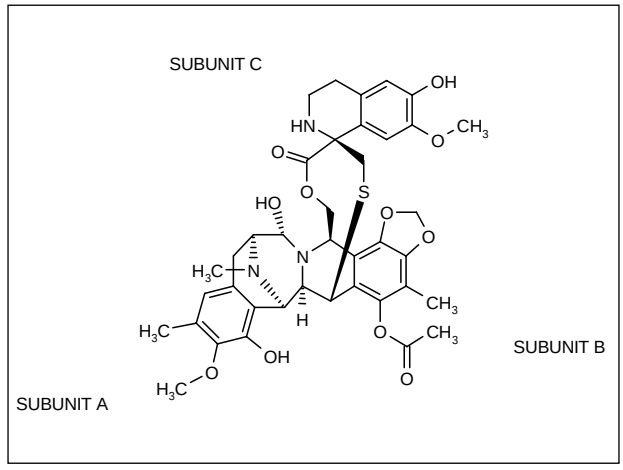

Fig. 3. Chemical structure of trabectedin.

while subunit C protrudes out of the minor groove perpendicular to the helix status (28).

The interaction between the protruding C ring with nuclear DNA binding proteins was thought to be one of the mechanisms responsible for the cytotoxic effects of trabectedin, although recently this hypothesis has been ruled out. In fact, a trabectedin analogue lacking the C subunit but with the same chemical structure has shown superior biological activities to those of trabectedin (29).

The binding of trabectedin to the minor groove of DNA induces a bending of the DNA towards the major groove. This is a unique feature of the drug as all the other DNA binding agents cause structural perturbation of the DNA by bending it towards the site of their interaction rather than away from it (*i.e.*, alkylating agents binding the major groove bend DNA towards the major groove while minor groove binders bend DNA towards the minor groove).

Trabectedin did not significantly inhibit the binding of sequence-specific transcriptional factors to DNA. As an exception, the serum response factor (SRF)/ternary complex factor (TCR) and the nuclear transcription factor Y (NF-Y) showed some degree of sensitivity to the drug (30). Further studies showed that trabectedin was able to induce a block in the transcription of inducible genes such as HSP70 and MDR-1 leading to the general conclusion that the drug could have an inhibitory effect on activated transcription rather than on constitutive gene transcription (31-33). Microarray gene expression analysis showed changes in gene expression induced by trabectedin treatment, although from the data available thus far, no evidence for a common mechanism of induction or inhibition of gene transcription has yet been found (34).

Regarding its mechanism of action, strong evidence has been produced on the influence of the DNA repair cellular system on trabectedin activity. Isogenic cell lines with specific DNA repair defects helped to determine that the mismatch repair system does not influence the activity of the drug, while a strong role is played by nucleotide excision repair (NER). NER-deficient cells are generally more sensitive to the action of alkylating agents such as cisplatin or to UV treatment. Intriguingly, trabectedin was less active in cells with NER defects, making it the only molecule to date having this interesting characteristic (35).

When the effects on cell cycle were analyzed, it was found that trabectedin induced a delay of cell progression from the G_1 to the G_2 phase, inhibition of DNA synthesis and a blockade in the G_2 phase. Experiments performed in synchronized cells showed that the drug seemed to be more active against cells present in the G_1 phase of the cell cycle than against cells in other phases (36).

Preclinical activity *in vitro* showed a very high potency of the drug and a broad spectrum of activity. A peculiar cytotoxicity against soft tissue sarcoma cell lines, where trabectedin was active at picomolar concentrations, has been reported (37). The *in vitro* activity was also confirmed *in vivo*. In fact, trabectedin treatment was very active against human tumors of different origin transplanted in nude mice (38, 39).

The distinct mechanism of action of the drug stimulated studies aimed at evaluating its activity in combination with other drugs. Synergistic activity was obtained when trabectedin was combined with cisplatin, with no evidence of sequence dependency. The synergism of the combination was confirmed *in vivo* in nude mice transplanted with different human tumors (40). The combination of trabectedin with docetaxel also showed synergism, with no increase in toxicity (41). Additive effects were observed when trabectedin was combined with 5-FU and camptothecin in some cell lines. An additive synergistic effect was found when doxorubicin was combined with trabectedin both *in vitro* and *in vivo*. This combination was also active against tumors where the single drugs showed no or only marginal activity (42).

Pharmacokinetic studies showed a high binding of trabectedin to plasma proteins with high clearance and a high volume of distribution. *In vitro* studies with rat and human microsomes showed extensive cytochrome P450-dependent metabolism of trabectedin.

Clinical trials with trabectedin have been conducted both in Europe and the U.S. Phase I studies were performed using different schedules including 1-h or 3-h infusions, continuous infusion (24-72 h) and daily doses for 5 consecutive days. The maximum tolerated dose (MTD), which was the recommended dose for phase II studies, was 1000-1110 $\mu g/m^2$ for the 1-h infusion schedule and could be increased to 1650-1800 $\mu g/m^2$ without any further modification of the toxicity profile for the 3-h infusion schedule (reviewed in 43). The MTD for the 24-h infusion schedule was 1800 $\mu g/m^2$. Dose escalation was discontinued at 1200 $\mu g/m^2$ with the 72-h continuous infusion schedule because of toxicities including grade 4 rhabdomyolysis, febrile neutropenia, thrombocytopenia and reversible acute renal failure. Even though the recommended dose was defined, this schedule was not recommended for further clinical development. In the repeated-administration schedule (daily 1-h infusion for 5 consecutive days every 3 weeks) the maximum administered dose was 380 $\mu g/m^2$. Overall and irrespective of the duration of the infusion, the main toxicities were an early, dose-dependent increase in transaminases, bilirubin and alkaline phosphatase, nausea and vomiting, and schedule-dependent myelosuppression which was more frequent after prolonged infusion. Objective responses were observed with all schedules of treatment and these promising results, especially those in soft tissue sarcoma (STS), prompted the development of a large phase II program in Europe and the U.S. A summary of the published phase II trials in soft tissue sarcoma is reported in Table I.

Overall, the phase II studies confirmed the phase I studies with regard to the low objective response rate, long response duration and high percentage of progression-free patients at 6 months. In particular, the antitumor activity of trabectidin in pretreated STS patients is similar to that reported for agents considered to be active. The ultimate role of trabectidin in the treatment of STS, however, needs to be tested and proven in the most

Table I: Phase II studies of trabectedin in soft tissue sarcomas.

Regimen (ref.)	Number prior CT cycles	Patients	Objective response (number)	Minor response
1500 μg/m^2 24-h inf. q3wks (60)	None	36 (34 evaluable)	6 (PR)	
	16 (44%) 1	36	1 (CR)	2
	20 (56%) ≥ 2		2 (PR)	
	(58% chemo + radiotherapy)			
1500 μg/m^2 24-h inf. q3wks (61)	1 combination or ≤ 2 single agents	26 (23 evaluable)	0	2
	≥ 3 single agents ≥ 2 combinations	28 (27 evaluable)	2 (PR)	2
1500 μg/m^2 24-h inf. q3wks (62)	1 cycle	44	3 (PR)	4
	2 cycles	55	6 (PR)	4
1500 μg/m^2 24-h inf. q3wks (63)	Median 3 cycles (range 1-11)	198 (55 evaluable)	1 (CR)	8
			5 (PR)	
1300-1500 μg/m^2 3-h inf. q3wks (64)		48	6	

CT = chemotherapy; CR = complete response; PR = partial response

favorable situation, *i.e.*, front-line in combination with anthracyclines. In this regard, phase I studies with anthracyclines have already been implemented. Positive results were also obtained in a phase II study in platinum/taxane-pretreated ovarian cancer patients, with an overall response rate of 43% in platinum-sensitive patients and an estimated median time to progression of 7.9 months (44). These results are comparable to those achieved with liposomal doxorubicin which is the recognized effective salvage treatment in this patient population. In the same study, 2 partial responses were reported in 30 platinum-resistant patients. Although less encouraging, this result is of interest in light of the experimental and preliminary clinical results suggesting a synergistic effect of the trabectedin-platinum combination in platinum-resistant disease.

New sequence-specific minor groove binders

Different laboratories have attempted to apply structure-activity studies to the design of compounds able to interact with the minor groove of DNA but, unlike the distamycin-like compounds, having a more pronounced specificity for GC-rich regions in the minor groove (45, 46). A number of compounds have been characterized for their *in vitro* and *in vivo* antitumor activity, some of which are now approaching the clinic.

SJG-136

One interesting compound is SJG-136, a novel pyrrolobenzodiazepine dimer belonging to the family of naturally occurring anticancer antibiotics comprising anthramycin, sibiromycin and tomaymycin (47). These compounds are able to form interstrand crosslinks and covalently bind the N2 position of guanine in the minor groove of DNA. This binding has been shown to block transcription in a sequence-specific manner (48).

SJG-136 was tested against the National Cancer Institute's panel of 60 cell lines and was found to share some features of DNA binding drugs (49). However, the data obtained by analyzing the gene expression patterns induced after drug treatment did not show any particular alignment with other anticancer agents, suggesting that the compound could have a peculiar mechanism of action.

In testing the *in vitro* activity of SJG-136 against leukemia and normal bone marrow cells, it was found that the concentration inhibiting the growth of leukemia cells was at least 10 times lower than that necessary to inhibit the growth of normal cells. The compound has shown very promising *in vivo* activity against a panel of human tumors and, interestingly, it was also active *in vivo* against cisplatin-resistant tumors.

DNA seems to be SJG-136's target. Indeed, the compound causes DNA crosslinks not only in naked DNA but also in cells and, more importantly, *in vivo* in tumors growing in nude mice receiving pharmacologically active doses of the compound. The cytotoxicity of the compound was reported to be p53-independent with a strong induction of apoptosis.

In vivo studies on the antitumor activity of SJG-136 showed that the compound was active against both relatively small and large tumors, causing tumor growth reduction in 9 of 10 human xenografts tested. The maximum activity was observed with intravenous bolus administration for 5 days. The preclinical results obtained for SJG-136 are very encouraging and largely fulfill the NCI criteria for predicting activity in phase II clinical trials, *i.e.*, that a compound should show antitumor activity *in vivo* in more than one-third of tumor models. The activity of SJG-136 is well above these criteria, and we are now awaiting the clinical results of the first phase I studies already in progress in the U.K. and the U.S.

Other DNA minor groove binding molecules

The well-known fluorescent DNA stains Hoechst 33258 and Hoechst 33342, which are minor groove binding ligands (50, 51), have been used as a starting

chemical structure to generate new analogues possessing superior activity. Methylproamine was one of these derivatives which showed very potent radioprotective activity (52). Other derivatives were synthesized with the aim of producing compounds with selective ability to inhibit the binding of transcription factors to DNA. The microgonotropens are one such class of compounds able to inhibit transcription factors/DNA complexes and some of them are also able to inhibit endogenous gene expression regulated by the transcription factors inhibited at the DNA level (53-55).

FR-900482 and FK-317 are compounds that crosslink proteins to DNA in the minor groove. In particular, they are able to crosslink HMG proteins to DNA, representing the first example of a drug able to crosslink a cancer-related protein to DNA in cells (56, 57).

MS-247 is a netropsin-like compound with an alkylating moiety attached. This compound showed high antitumor activity against human tumor xenografts transplanted in nude mice, and the mechanism of action indicated that it binds DNA at the same regions bound by Hoechst 33342 (58, 59).

Other structures are still being evaluated for their biological activity and the results are expected in the next few years.

Conclusions

Minor groove binding agents have been tested for many years for their potential antitumor activity. The extremely high antitumor activity observed at the preclinical level has been somewhat obscured by the unexpectedly high toxicity observed at the clinical level. Many of the drugs initially entering clinical studies were in fact withdrawn due to dose-limiting adverse effects. Since then, a number of studies and methodologies have been employed for the discovery of minor groove DNA binding agents combining high antitumor potency with low cytoxicity, particularly against myeloid cells. New screening systems have been used with this aim in mind, and new compounds are now being evaluated in clinical studies and have shown acceptable toxicity. An interesting feature of the DNA minor groove binding class of drugs is the possibility to combine them with other anticancer agents, taking advantage of their peculiar mechanism of action. For some drugs such as brostallicin and trabectedin, evidence for clinical activity has been shown, thus underscoring the need for developing and testing new minor groove binding compounds.

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