Brostallicin: a new concept in minor groove DNA binder development

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Brostallicin is a bromoacryloyl derivative of distamycin A, which has shown very promising preclinical activity against a variety of human tumors both *in vitro* and *in vivo*. The drug has a limited toxicity towards bone marrow precursor cells *in vitro* resulting in a therapeutic index much higher than those achieved with other distamycin A derivatives. It retains activity against cancer cells resistant to alkylating agents, topoisomerase I inhibitors and cells with mismatch repair deficiency. Brostallicin has a peculiar mechanism of action involving activation upon binding to glutathione (GSH) catalyzed by glutathione-S-transferase (GST). As a consequence, cells expressing relatively high GST/GSH levels are more susceptible to treatment with brostallicin. Considering that increased levels of GST/GSH are often found in human tumors, this could represent an advantage for the drug in the clinic. Initial clinical studies indicate the tolerability of the drug and allow the determination of the optimal dose for subsequent studies. Some partial response were obtained in these initial phase I studies. Altogether, the results suggest brostallicin to be a new promising anticancer agent with a new mechanism of action. It also raises the possibility to use it in combination with other anticancer drugs currently used. *Anti-Cancer Drugs* 15:1–6 © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:1–6

Keywords: brostallicin, minor groove DNA binder

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Received 12 August 2003 **Revised form accepted** 8 September 2003

Introduction

DNA minor groove-binding anticancer agents (MGB) are a class of compounds which have shown very high antitumor activity both *in vitro* and *in vivo* in different experimental tumor systems. At the molecular level, their mode of action is largely unknown. However, data in the literature suggest that this class of compound acts in a completely different fashion when compared to conventional DNA-interacting agents. Despite the fact that MGB are generally less reactive towards DNA than major groove binders, they are extremely sequence specific in alkylating DNA [1]. Loss of alkylation ability is associated with a dramatic loss of cytotoxicity in *in vitro* models, suggesting that their mechanism of action is tightly related to their covalent sequence-specific DNA interactions [2]. At variance from what has been previously reported for major groove binders, MGB cytotoxicity is not related to the induction of DNA inter/intrastrand cross-links or DNA protein cross-links.

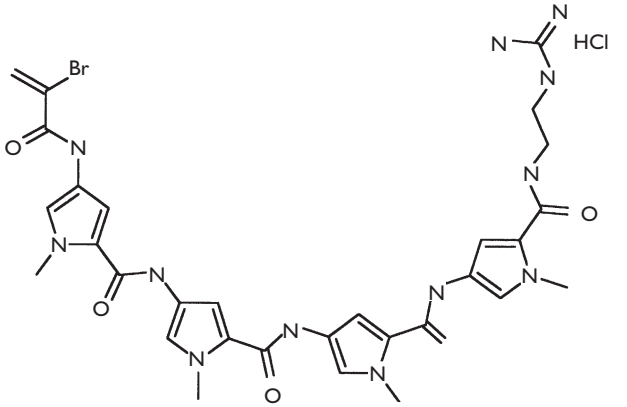
In spite of the very interesting preclinical profile, the clinical use of MGB is still very limited. While mitomycin C is approved and ET-743 (Yondelis) is under testing, the development of bizelesin, carzelesin, aldozelesin and tallimustine has been discontinued due to dose-limiting myelotoxicity. The search for novel MGB with an increased therapeutic index has continued, and brostallicin, a new analog with a unique and novel mechanism of action, has been recently identified and tested in cancer patients.

Distamycin-derived α-bromoacrylic derivatives: mechanism of action studies

Brostallicin is an α-bromoacrylamido derivative of a four pyrrole distamycin (DST)-like frame in which the amidine is replaced by a guanidine moiety (Fig. 1). It is structurally related to the compound PNU-151807 [3], which may be considered the first lead of the bromoacrylic derivative MGB. PNU-151807 showed *in vitro* cytotoxicity and *in vivo* antitumor activity significantly higher than that of the DST analog tallimustine (TAM) [4]. Differently from TAM, PNU-151807 was able to bind non-covalently to DNA minor groove TA-rich regions, but was unable to alkylate DNA in classical *in vitro* assays. The activity profile of PNU-151807 and its apparently unusual mechanistic features prompted the synthesis of a series of new halogenoacrylic derivatives of DST and congeners, implying modification of the DST frame and of the amidino moiety [5,6]. Among these DST derivatives, brostallicin was selected for further studies aimed at identifying the mechanism of action of this new class of compounds.

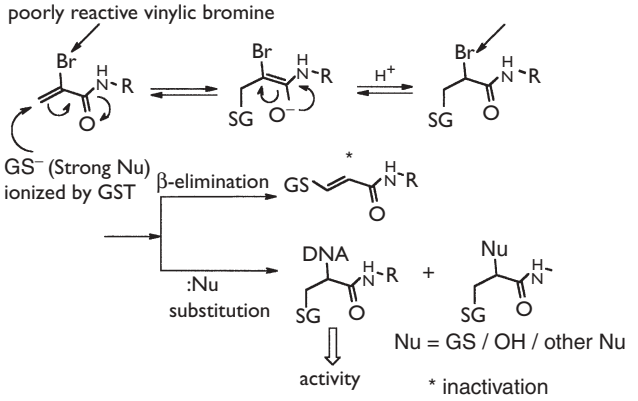
0959-4973 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000109326.56830.53

Fig. 1



Chemical structure of brostallicin.

Fig. 2



Putative role of GSH and GST in the interaction between brostallicin and DNA.

From a mechanistic point of view, α-bromo- and α-chloroacrylamido derivatives show a significant cytotoxicity, while the α-fluoroacrylamido and acrylamido derivatives are inactive. These data indicate that the reactivity of the α-halogenoacrylic moiety plays a key role in cytotoxicity. Brostallicin showed an increased activity against melphalan-resistant L1210 cells (L210/L-PAM), which are characterized by high levels of glutathione (GSH) [7], compared to wild-type L1210 cells (IC_{50} 0.46 and 1.45 ng/ml, respectively) in conditions where the cytotoxicity of conventional antitumor agents is either unaffected or reduced [8].

This finding, together with the importance of the halogenoacrylic moiety, led to the generation of the mechanistic hypothesis that, inside the cell and in the presence of an intracellular reactive nucleophilic species such as GSH, α-bromoacrylic derivatives like brostallicin could perform a first-step Michael-type attack followed by a further reaction leading to a second nucleophilic substitution, leading to alkylation of DNA nucleophilic functions [9] (Fig. 2).

The consequence is that brostallicin alkylates DNA only in the presence of cellular thiols (particularly GSH) whose intracellular concentration is in the millimolar range [10]. This hypothesis has been confirmed experimentally [9,11].

Agarose gel electrophoresis shows that brostallicin (and parent compound PNU-151807) induces the change of plasmidic DNA (plasmid pUC18/19 containing a sequence 5′-TTTTTTGA-3′ and two sequences 3′-TTTTTGA-3′) from the supercoiled to the circular form (nicking), only in the presence of GSH. Conversely, the inactive α-fluoroacrylic analog of brostallicin is unable to relax plasmidic supercoiled DNA in the presence of GSH [9,11].

The mechanism of brostallicin DNA interactions has been further analyzed by Taq polymerase stop assay, in comparison to TAM, with or without GSH and glutathione-S-transferase (GST). As previously reported [12], TAM retained its high sequence specificity in alkylating DNA at the target hexamer (5′-TTTTGA). In the case of brostallicin, the distamycin-like frame drives the compound towards its interaction in the minor groove of DNA [13], but brostallicin *per se* is completely unable to produce any alkylation in any of the selected DNA-interacting regions. When GST/GSH mixture is added to the reaction, the compound binds covalently AT-rich regions on DNA with a sequence specificity different from that previously reported for TAM [13].

In vitro and *in vivo* antitumor activity

Brostallicin shows potent *in vitro* cytotoxic activity against tumor cells with IC_{50} values in the low nanomolar range, circumvents resistance to alkylating agents and camptothecins, but not the MDR phenotype [14], and maintains, unlike other MGB, efficacy on mismatch repair-deficient cells [13]. The latter finding suggests potential efficacy on mismatch repair-defective tumors, e.g. hereditary non-polyposis colon cancer [15]. Brostallicin is 20-fold more active than TAM in inducing apoptosis in A2780 ovarian carcinoma cells. Its cytotoxicity/myelotoxicity ratio is outstanding, i.e. its mean IC_{50} against a series of tumor cell lines is about 80 times lower than that on human CFU-GM hematopoietic progenitors cells [16].

Brostallicin has broad antitumor activity in animal models. On human solid tumors xenografted in nude mice, non-toxic drug levels (0.52–0.78 mg/kg/day in three

or four i.v. administrations) are active against human ovarian, renal, prostatic, lung and colon carcinomas.

The antitumor activity of brostallicin is increased in tumor cells with higher GSH and/or GST levels

Brostallicin was found more active against L-PAM-resistant L1210 cells (expressing high GSH levels) than against parental cells (with relatively lower GSH content) (Table 1). The observation that the inhibition of GSH formation by means of buthionine sulfoximine (BSO, a specific inhibitor of γ-glutamylcysteine synthetase controlling *de novo* GSH biosynthesis), leads to a significant decrease of brostallicin cytotoxic and apoptotic effects on A2780 cells (Table 2) further supports the initial findings on L-PAM-resistant L1210 cells.

Preclinical proof of the importance of the GST/GSH system for brostallicin antitumor activity has been obtained. When human GST-π cDNA was transfected into A2780 cells and four clones of A2780 with different expression levels of GST-π were generated (i.e. two clones with high and two clones with low GST-π expression), a 2- to 3-fold increase in GST-π levels resulted in a 2- to 3-fold increase in cytotoxic activity (Table 1). These results were further confirmed on GST-π-transfected human breast carcinoma cells (MCF-7). Brostallicin showed 5.8 times increased cytotoxicity on GST-π-transfected versus empty vector-transfected cells with low GST-π expression. To complete this experiment with *in vivo* data, A2780 clones were implanted into nude mice. The antitumor activity of brostallicin was higher in the GST-π overexpressing tumors compared to tumors with lower GST-π expression, without increased toxicity [11].

Finally, the presence of GST-π significantly enhances the reaction of brostallicin with GSH, while the reaction with GSH alone under the same conditions is negligible [11]. Thus, GSH may affect both the mechanism of brostallicin–DNA interaction and its antitumor activity, and this underlines the novelty of brostallicin in comparison with TAM and other MGBs. In particular, a possible advantage in combination therapies with classical alkylating drugs that induce GSH/GST overexpression or a wider therapeutic index for tumors over-expressing GST-π can be expected. Due to the apparent GSH/GST activation, brostallicin may be considered to belong to the wide class of activated cytotoxic drugs, which has been recently reviewed [17].

Table 1 ***In vitro*** **cytotoxicity of brostallicin on tumor cells with different GSH/GST content**

Cell Line	GSH or GST level[c]	*In vitro* cytotoxicity (IC_{50} ng/ml)
L1210/L-PAM[a]	25.8	0.46
L1210	7.7	1.45
A2780/GST-π clone 8[b]	32.1	50.9
A2780/GST-π clone 7	26	63.4
A2780/GST-π clone 16	14	145
A2780/GST-π clone 2	11	>200
MCF-7/pMTG-5[b]	77.9	78.6
MCF-7 neo	3.3	462.7

[a]Cells incubated with brostallicin for 48 h.
[b]Cells incubated with brostallicin for 1 h.
[c]GSH (nmol/10^6cells) for L1210 cell sublines, GST (nmol/min/mg of proteins) for A2780 and MCF-7 cell sublines, normal and GST-π transfected.

In vivo activity in combination with other anticancer agents

A clear therapeutic gain is observed in preclinical models when brostallicin is combined with anticancer agents such as cisplatin, doxorubicin, CPT-11 and taxotere.

The cisplatin/brostallicin interaction is dependent on the sequence of delivery, leading to a more than an additive antitumor effect, without additional toxicity, only when cisplatin is given before brostallicin This sequence-dependent activity can be explained by the evidence that treatment of cancer cells with cisplatin resulted in an increased expression of GST [18,19]. The antitumor effect of CPT-11 is significantly enhanced by brostallicin co-treatment. The maximum tolerated dose of each agent can be administered without additional toxicity. In the *in vivo* systemic L1210 murine leukemia model a more than additive antitumor effect is observed when doxorubicin and brostallicin are administered sequentially. Additivity is observed when the brostallicin and taxotere combination is tested simultaneously.

These results indicate that, although the precise mechanism of drug–drug interaction is not yet elucidated, brostallicin is not only an active compound in single-agent therapy, but is also a promising agent in combination with other anticancer drugs [20].

Pharmacokinetics

The preclinical pharmacokinetics of brostallicin have been investigated in mice and monkeys. In both animal

Table 2 Cytotoxicity and apoptotic effects of brostallicin on A2780 cells treated with BSO

Treatment	Dose	Growth inhibition (%)	Apoptosis (%)
Brostallicin	300 ng/ml	43 ± 2.5	17.5 ± 6.5
	1000 ng/ml	64.5 ± 14.5	49.5 ± 15.5
	3000 ng/ml	77.5 ± 6.5	74.5 ± 15.5
Brostallicin + BSO[b]	300 ng/ml	31.5 ± 2.5	1.5 ± 0.5
	1000 ng/ml	33 ± 11	5.5 ± 2.5
	3000 ng/ml	38 ± 7	11.5 ± 5.5
BSO[c] 24/48 h	0.1/0.1 mM	1 ± 1/2.5 ± 2.5	0/0

[a]Cells incubated with the compound for 24 h. Cell growth inhibition determined by counting surviving cells and apoptosis determined by morphological examination. Values are the means ± SE of at least three independent experiments each consisting of six replicates.
[b]Cells exposed to BSO (0.1 mM) for 24 h before and during brostallicin treatment.
[c]Cells exposed to BSO (0.1 mM) for 24 h.

species, brostallicin shows a rapid elimination with a terminal half-life in the range of 0.4–3.6 h, moderate clearance and moderate distribution into tissues. The systemic exposure following repeated dosing in mice and monkeys is somewhat higher than what would be predicted from the half-life of brostallicin after single administration, suggesting some degree of time dependency. No major gender differences are evident. The main route of elimination of drug-related radioactivity in mice and monkeys is fecal, as shown by excretory balance studies carried out with the ^{14}C-labeled compound [21]. The metabolism was investigated *in vitro* using rat hepatocytes as well as mouse, rat, dog, monkey and human liver microsomes. The primary biotransformation route observed is debromination, with replacement of the bromine atom by hydrogen. The debrominated metabolite (PNU-230858) is several times less cytotoxic than the parent compound and inactive *in vivo*. In microsomal incubation tests, the turnover of brostallicin is low without major differences between the different species and humans. The *in vivo* metabolism has been investigated in mice and monkeys after i.v. administration of [^{14}C]brostallicin. Trace amounts of the previously identified PNU-230858 metabolite could be detected in mouse urine. The other metabolites detected in both mouse and monkey urine and mouse bile result from the conjugation of brostallicin with GSH. All the identified metabolites are metabolized at the α-bromoacrylic moiety and lose the bromine atom, thus reinforcing the hypothesis of the first-step Michel-type attack previously discussed.

Toxicology

Brostallicin is characterized by a radically reduced myelotoxicity compared to TAM and other MGBs [22] in all tested species, and, in particular, on human progenitor cells, as demonstrated in the *in vitro* clonogenic assay.

In order to evaluate the toxicological profile of i.v. brostallicin, single- and repeated-dose (5-day, weekly and chronic cyclic administration) toxicity studies were performed in mice and monkeys.

In the single-dose studies, brostallicin induces a dose-related, reversible myelotoxicity in both species. Myelosuppression is the cause of death in mice but no deaths are observed in monkeys at the doses administered. Minor targets seen are the intestine and kidney in mice, and the liver in both species. All changes show a complete or almost complete regression during the recovery period.

The LD_{10} values in mice are 3.54 and 2.86 mg/kg in males and females, respectively (10.6 and 8.6 mg/m^2). The maximum non-lethal doses (MNLDs) in the single-dose studies are 3.1 and 2.5 mg/kg (9.3 and 7.5 mg/m^2) in male and female mice, respectively, and higher than 2.0 mg/kg (24.6 mg/m^2) in monkeys of both sexes.

Five-day repeated i.v. administration of brostallicin induces myelosuppression, which is the cause of death in both animal species. Again, the GI tract, liver and kidney are minor targets of the compound. All changes totally or partially regress during the 4-week recovery period. Repeated daily doses are associated with increased toxicity in the hemolymphopoietic system, but not in the liver and kidney. The MNLDs in the 5-day studies are 0.20 mg/kg/day (cumulative dose 1.0 mg/kg) in mice and 0.10 mg/kg/day (cumulative dose 0.5 mg/kg) in monkeys.

The results of an exploratory study in monkeys with weekly administration suggest that repeated administration with longer intervals between doses should be better tolerated than daily administration, as also show by the MNLD, which is slightly higher than that of the 5-day study (0.25 versus 0.10 mg/kg/day).

Chronic cyclic studies were performed administering the compound once every 3 weeks for a total of nine cycles in both species, with MNLDs of 0.9 (cumulative dose 8.1) and 0.5 mg/kg/cycle (cumulative dose 4.5 mg/kg) in mice and monkeys, respectively.

Brostallicin is genotoxic in the Ames test, as expected for this class of agents.

Finally, an embryo–fetal development study has been performed in female mice by daily administration (0.25–1 mg/kg/day) on gestation days 8.5 and 9.5. Based on the results of the study, there was not a NOEL (no observed effect level) for development toxicity and teratogenicity is observed at the highest dose of 1 mg/kg/day.

Clinical studies with brostallicin

Two phase I studies were carried out in a total of 41 adult patients with advanced/metastatic solid tumors. The first study explored an every-3-week (q3w) schedule of administration [23]. The other one tested a weekly × 3 followed by 1 week of rest schedule [24]. In both studies, brostallicin was administered i.v. over 10 min. The maximum tolerated doses were 10 mg/m^2 in the q3w study and 2.4 mg/m^2/week in the weekly study. Grade 4 neutropenia and thrombocytopenia were the dose-limiting toxicities in both studies. No double nadir was observed for either platelets or neutrophils. In the q3w study, the neutrophil and platelet nadirs occurred around day 14 and a complete recovery was observed in most patients by day 21. No cumulative hematologic toxicity was reported. Anti-emetic prophylaxis was required.

Table 3 Overview of phase I studies with brostallicin

Schedule	No. of patients	Dose range	Maximum tolerated dose	Dose limiting toxicity	Other toxicities	Antitumor activity
q3w	27	0.85–15 mg/m^2	10 mg/m^2	neutropenia; thrombocytopenia	nausea; vomiting	1 PR (GIST), 5 SD, 20 PD, 1 NE
Weekly × 3 (+1 week rest)	14	0.3–4.8 mg/m^2/week	2.4 mg/m^2/week	neutropenia; thrombocytopenia	nausea; vomiting	6 SD, 7 PD, 1 NE

In the q3w study, a long-lasting partial response (PR) was documented after 6 cycles in a female patient with extensive liver metastases due to a gastrointestinal stroma tumor (GIST) treated at a dose of 5.1 mg/m^2. Phase I studies are summarized in Table 3.

The pharmacokinetics of brostallicin has been determined in both phase I studies. The plasma concentration showed a rapid decline at the end of the infusion with the terminal half-life being about 5 h. Tissue distribution was moderate. The clearance was low (about 20–30% of the hepatic blood flow). The systemic exposure and the plasma concentration at the end of the infusion increased linearly with the dose. No time dependence was generally observed.

Phase II studies with the q3w schedule are currently ongoing in both solid and hematological tumors.

Conclusions

The very promising preclinical antitumor activity of MGB led to the development of new analogs possessing high antitumor activity associated with low toxicity. *In vitro*, brostallicin has a much more favorable therapeutic index than previous MGBs, as indicated by the mild toxicity towards normal hematopoietic cells growing *in vitro*. From a mechanistic point of view, brostallicin presents the unique feature of maintaining anticancer activity in tumors resistant to different classes of antitumor agents and showing an increased activity against tumors expressing high levels of GST/GSH. Increased expression of the GST/GSH system is often found in human tumors and at time of relapse after a first line of treatment with classical alkylating agents. This could represent an advantage for brostallicin (as indicated by several *in vitro* and *in vivo* experiments) and lead to an alternative treatment for tumors refractory to alkylating agents.

From a molecular point of view, no data are at present available on the molecular targets responsible for brostallicin cytotoxic activity. However, the finding that by increasing the number of pyrrole rings linked to the α-bromoacrylic moiety (and thus increasing compound affinity for DNA AT-rich regions) compounds are obtained with increased toxicity [9] strongly supports the idea that upon being activated by GSH/GST, the molecule exerts its activity towards the DNA. To explore mechanism-based treatment modes which may allow exploitation of molecular knowledge for improved clinical use, the recent finding that GST-π is present within the nucleus and that its levels increase upon exposure to DNA-damaging agents, like doxorubicin and cisplatin [25], makes brostallicin a good candidate for combination therapy with different anticancer agents.

In vivo tests have shown that the combination of brostallicin with other anticancer agents, such as taxanes, cisplatin, anthracyclines and topoisomerase I inhibitors is feasible, shows more than additive activity (i.e. cisplatin and CPT-11), and does not result in increased toxicity.

The mechanism of action of brostallicin opens the possibility to test the clinical activity of this compound according to tumor GSH/GST status. Methods for patient selection on the basis of patients and/or tumor GSH/GST level/expression are being explored [26].

An additional, attractive therapeutic application is represented by tumors with low GST levels due to hypermethylation of GST gene promoter regions. This is particularly relevant for prostate carcinoma, where more than 90% of tumors are characterized by this genetic alteration which lead to GST gene silencing [27].

Combination of brostallicin with agents able to revert the hypermethylation and allowing the re-expression of GST-π gene could therefore result in a tumor-selective synergistic activity.

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