Brostallicin, a Novel Anticancer Agent Whose Activity Is Enhanced upon Binding to Glutathione

Cristina Geroni, Sergio Marchini, Paolo Cozzi, Emanuela Galliera, Enzio Ragg, Tina Colombo, Rosangela Battaglia, Martin Howard, Maurizio D’Incalci, and Massimo Broggiini

ABSTRACT

Brostallicin (PNU-166196) is a synthetic α-bromoacryloyl, second-generation DNA minor groove binder structurally related to distamycin A, presently in Phase II trials in Europe and the United States. The compound shows broad antitumor activity in preclinical models and dramatically reduced in vitro myelotoxicity in human hematopoietic progenitor cells compared with that of other minor groove binders. Brostallicin showed a 3-fold higher activity in melphalan-resistant L1210 murine leukemia cells than in the parental line (IC50 = 0.46 and 1.45 ng/ml, respectively) under conditions in which the cytotoxicity of conventional antitumor agents was either unaffected or reduced. This melphalan-resistant cell line has increased levels of glutathione (GSH) in comparison with the parental cells. Conversely, GSH depletion by buthionine sulfoximine in a human ovarian carcinoma cell line (A2780) significantly decreased both the cytotoxic and the proapoptotic effects of brostallicin. In one experiment, human glutathione S-transferase π (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 2-3-fold increase in GST-π levels resulted in a 2-3-fold increase in cytotoxic activity of brostallicin. Similar results were obtained for GST-π-transfected human breast carcinoma cells (MCF-7). Brostallicin showed 5.8-fold increased cytotoxicity in GST-π-transfected versus empty vector-transfected cells with low GST-π expression. In an in vitro experiment, A2780 clones were implanted into nude mice. The antitumor activity of brostallicin was higher in the GST-π-overexpressing tumors without increased toxicity. Regarding the mechanism of action, brostallicin interacts reversibly with the DNA minor groove TA-rich sequences but appears unreactive in classical mustards, DDP, and anthracyclines (20–23), and several tumor multiresistant cell lines are also ~100 times more sensitive than murine bone marrow cells (11). The comparative assessment of the sensitivity of human and murine bone marrow cells to other distamycin derivatives showed that different analogues were much less toxic against human cells. PNU-151807, a bromoaacroyl derivative of distamycin A, was the first compound of this class studied and showed several interesting features indicative of a mechanism of action different from that of tallimustine (12–15). Another member of this class, brostallicin (PNU-166196; N-[5-[5-[2-(aminoiminomethyl)amino]ethyl]amino]carbonyl]-1-methyl-1H-pyrrol-3-yl)aminocarbonyl]-1-methyl-1H-pyrrol-3-yl)aminocarbonyl]-1-methyl-1H-pyrrol-3-yl)-4-[4-(2-bromo-1-oxo-2-propenyl)amino]-1-methyl-1H-pyrrol-2-yl(carbonyl) amino]-methyl-1H-pyrrole-2-carboxamide; Fig. 1), was selected for clinical development because of its outstanding antitumor activity in several preclinical tests as well as its favorable toxicity profile (3, 14, 16–18). Characterizing the pharmacological properties of brostallicin, we obtained evidences that the GSH/GST system may play a peculiar role in determining the sensitivity of cells to this drug. The biological and biochemical studies reported here support the idea that brostallicin activity is increased in the presence of high GSH/GST levels and that these findings have potential value in cancer treatment (17–19). In fact, high levels of GSH and GST have been reported to play a role in the resistance of tumor cells to different anticancer drugs, such as classical mustards, DDP, and anthraclyclines (20–23), and several tumor types display increased levels of GSH and/or GST with respect to normal tissues (23–27). These facts underline the novelty of brostallicin, presently undergoing Phase II clinical trials.

INTRODUCTION

MGBs represent a class of anticancer agents whose DNA sequence specificity may lead to a high selectivity of action (1–3). Representative compounds of this class are the antitumor agents derived from CC-1065 (4–6) and the nitrogen mustard derivative of distamycin A, tallimustine (7, 8). Although these compounds were found to be very active against experimental tumors unresponsive to other antineoplastic agents, they did not proceed in clinical studies because of their severe dose-limiting myelotoxicity, which precluded further development (9, 10). For tallimustine, the severe bone marrow toxicity was much more marked in humans than in rodents, where its antitumor activity was demonstrated. The interspecies differences were probably mainly attributable to intrinsic cellular differences in sensitivity as demonstrated by the fact that in vitro human bone marrow progenitor cells were also ~100 times more sensitive than murine bone marrow cells (11). The comparative assessment of the sensitivity of human and murine bone marrow cells to other distamycin derivatives showed that different analogues were much less toxic against human cells. PNU-151807, a bromoaacroyl derivative of distamycin A, was the first compound of this class studied and showed several interesting features indicative of a mechanism of action different from that of tallimustine (12–15). Another member of this class, brostallicin, was selected for clinical development because of its outstanding antitumor activity in several preclinical tests as well as its favorable toxicity profile (3, 14, 16–18). Characterizing the pharmacological properties of brostallicin, we obtained evidences that the GSH/GST system may play a peculiar role in determining the sensitivity of cells to this drug. The biological and biochemical studies reported here support the idea that brostallicin activity is increased in the presence of high GSH/GST levels and that these findings have potential value in cancer treatment (17–19). In fact, high levels of GSH and GST have been reported to play a role in the resistance of tumor cells to different anticancer drugs, such as classical mustards, DDP, and anthraclyclines (20–23), and several tumor types display increased levels of GSH and/or GST with respect to normal tissues (23–27). These facts underline the novelty of brostallicin, presently undergoing Phase II clinical trials.

MATERIALS AND METHODS

Drugs. Brostallicin and tallimustine were synthesized by Pharmacia Corporation (Milan, Italy). DDP, BSO, and L-PAM were purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved and diluted just before use.

Cell Lines and Drug Sensitivity. The murine lymphocytic leukemia L1210, the subline resistant to L-PAM (L1210/L-PAM), and the human ovarian carcinoma A2780 cell lines were grown in RPMI 1640 (Life Technologies). A2780 clones overexpressing the human GST-π gene were obtained after calcium phosphate-mediated transfection of parental cells with the human GST-π cDNA and selection in medium containing 500 μg/ml G418. The human breast carcinoma cell line MCF-7 and its clone overexpressing the human GST-π gene were grown as reported (28). All cell lines were maintained at 37°C, 5% CO2 in medium supplemented with 10% FCS. Drug cytotoxicity against L1210 and L1210/L-PAM was evaluated by counting surviving cells on a Coulter ZM Cell Counter (Coulter Electronics, Hialeah,
Drug-induced cytotoxicity in A2780 and MCF-7 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test in 96-well plates. Exponentially growing cells were seeded and exposed to various drug concentrations. The antiproliferative activity of the drug was calculated from dose-response curves and expressed as IC50.

Apoptosis in A2780 cells was evaluated by fluorescence microscopy (29). Floating cells were collected the end of the treatment, washed in PBS, and fixed in 70% ice-cold ethanol. Cell pellets were stained with 50 μg/ml propidium iodide, 0.001% NP40, and 60 units/ml RNase and stored in the dark for 30 min at 37°C. Cells were then centrifuged and resuspended in 50 μl of PBS. At least 600 cells randomly chosen from two independent smears were examined for their nuclear morphology changes (chromatin condensation and DNA fragmentation).

**Measurement of GSH and GST Activity.** Total GSH was measured from cells growing in culture as described previously (30). Total GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate (31). Reactions were performed with cytosolic extracts, and the conversion of 1-chloro-2,4-dinitrobenzene by GST was measured with a spectrophotometer. The data are expressed as nmol of diethyleneglycolurathione formed/min/mg of protein at 37°C, using the extinction coefficient 9.6 mM−1cm−1 (31).

**Brostallicin-GST Interaction Studies.** Incubations contained 10 μM brostallicin; 1 mM GSH; 2.5 μM/mL GST (human expressed A1-1, M1-1, and P1-1; Oxford Biomedical Research Inc, Oxford MI); and BSA (in control only; 2.5 μg/ml; Sigma Chemical Co.) in phosphate buffer (pH 6.5). After incubation for 5 min, the chemical reaction and enzymatic metabolism were stopped by the addition of acetonitrile containing N-ethylmaleimide (Fluka; Ref. 32). Incubations were performed in triplicate, and the amount of remaining brostallicin was measured in each incubation by high-performance liquid chromatography with UV detection. Results are expressed as percentage of brostallicin consumed in the incubations.

A similar experiment was performed to determine the intrinsiccleanness of brostallicin in the presence of the different GST isoenzymes. Brostallicin (1 μM) was incubated with GSH (1 mM) and the different GST isoenzymes (2.5 μg/mL). Acetonitrile containing N-ethylmaleimide was added to incubations to stop the chemical reaction and enzymatic metabolism after 2, 5, 10, 20, and 30 min of incubation. The amount of remaining brostallicin was determined by high-performance liquid chromatography with mass spectrometric detection. The CLint was determined from the observed half-life of brostallicin.

**In Vivo Activity.** Female nude Swiss NCr NuNu mice (Charles River Calco, Lecco, Italy; 4–6 weeks of age; weight, 20–25 g) were used in experiments with human tumors. Mice were maintained under specific pathogen-free conditions and provided sterile food and water ad libitum. A total of 106 cells/mouse, derived from A2780 clones, were implanted s.c. into the left flank of recipient mice. When the tumor was palpable (200 mg), animals were divided randomly into test groups consisting of at least six mice each (day 0).

**RESULTS**

The cytotoxic activity of brostallicin was initially evaluated in the murine leukemia L1210 subline resistant to L-PAM (L1210/L-PAM), characterized by higher GSH levels compared with the parental cell line (Table 1). Brostallicin was 3-fold more cytotoxic in L-PAM resistant cells under conditions in which L-PAM was 5-fold less active and the minor groove DNA alkylation tallimustine was equally active in sensitive and resistant cells. Further evidence for a role of GSH in modulating the activity of brostallicin was obtained by determining the influence of pretreatment with the GSH inhibitor BSO on the susceptibility of A2780 human ovarian cells to cytotoxicity and apoptosis induced by the drug. Depletion of GSH by BSO significantly decreased the efficacy of brostallicin (Table 2).

To explain the role of GSH in the enhancement of the in vitro activity of brostallicin and on the basis of the electrophilic reactivity of its α-bromoacrylic moiety, we speculated that GSH, as an intracellular reactive nucleophilic species, could react with the α-bromoacrylamide moiety, leading to the formation of a highly reactive GSH complex representing the real effective agent of brostallicin activity. We therefore performed a series of experiments with recombinant human GST-P1-1, -M1-1, and -A1-1 (α, μ, and π isoenzymes, respectively) aimed at checking the roles of GSH and GST in brostallicin activity and mechanism of action.

As shown in Table 3, coincubation of brostallicin and GSH alone did not result in a significant formation of the complex. Conversely, the presence of GST enhanced the reaction, and the GST-P1-1 and GST-M1-1 isoenzymes were stronger activators than the GST-A1-1 isoenzyme (46, 50, and 23% formation of GSH-brostallicin complex, respectively, after 5-min incubation).

These results were confirmed by performing an experiment to determine the CLint of brostallicin in the presence of the different GST isoenzymes. The GST-P1-1 and GST-M1-1 isoenzymes gave CLint values of 337 and 643 ml/min/mg protein, respectively, compared with 0.60 ml/min/mg protein observed in the absence of GST.

**Table 1 In vitro cell growth inhibition induced by brostallicin, tallimustine, and L-PAM in L1210 and L1210/L-PAM cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>GSH content (mmol/mg cells)</th>
<th>IC50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>7.7</td>
<td>1.45 ± 0.4</td>
</tr>
<tr>
<td>L1210/L-PAM</td>
<td>25.8</td>
<td>0.46 ± 0.2</td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
<td>3.6 ± 0.2</td>
</tr>
</tbody>
</table>

*Cells were incubated with the compound for 48 h. Cell growth was determined as reported in “Materials and Methods.” Values are the means ± SE of at least three independent experiments, each consisting of six replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Growth inhibition (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brostallicin</td>
<td>300 ng/ml</td>
<td>43 ± 2.5</td>
<td>17.5 ± 6.5</td>
</tr>
<tr>
<td>1000 ng/ml</td>
<td>64.5 ± 14.5</td>
<td>49.5 ± 15.5</td>
<td></td>
</tr>
<tr>
<td>3000 ng/ml</td>
<td>77.5 ± 6.5</td>
<td>74.5 ± 15.5</td>
<td></td>
</tr>
<tr>
<td>Brostallicin + 0.1 mM BSO</td>
<td>300 ng/ml</td>
<td>31.5 ± 2.5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>1000 ng/ml</td>
<td>33 ± 11</td>
<td>5.5 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>3000 ng/ml</td>
<td>38 ± 7</td>
<td>11.5 ± 5.5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BSO</th>
<th>24 h</th>
<th>0.1 mM</th>
<th>1 ± 1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td>0.1 mM</td>
<td>2.5 ± 2.5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were incubated with the compound for 24 h. Values are the means ± SE of at least three independent experiments, each consisting of six replicates.

*Cells exposed to BSO for 24 h before and during brostallicin treatment.
with a value of 5 ml/min/mg protein for the GST-A1-1 isoenzyme (Table 4).

To further corroborate and confirm that brostallicin antitumor activity was indeed enhanced in cells expressing high GSH/GST levels, we transplanted A2780 human ovarian carcinoma cells with the human GST-σ isoenzyme, and clones showing high GST activity were selected for further studies. Two clones overexpressing GST (A2780/GST 7 and A2780/GST 8; GST activity, 25.0 and 30.7 nmol/min/mg protein, respectively) were compared with one clone with low GST (A2780/GST 16; GST content, 13.4 nmol/min/mg protein). Concentration-response curves (Fig. 2A) indicated that brostallicin is significantly more active in A2780 clones expressing higher GST levels. Different from brostallicin, DDP was equally cytotoxic in A2780 clones with high or low GST-σ activity (data not shown).

Similar results were obtained in the MCF-7 human breast carcinoma cell line and in one previously characterized clone transfected with the human GST-σ (28) and expressing five times more GST than parental cells. Again, GST-σ-overexpressing cells were more susceptible to the cytotoxicity of brostallicin than the empty vector-transfected MCF-7 cells (Fig. 2B).

The A2780 clones with different GST-σ content were implanted in nude mice, and the antitumor activity of brostallicin was evaluated in vivo (Fig. 3). Brostallicin showed greater activity in the GST-σ-overexpressing tumors (A2780/GST 7 and A2780/GST 8) than in the tumors expressing normal levels of the enzyme (A2780/GST 16) without increased toxicity. In two clones (A2780/GST 8 and A2780/GST 16), we compared the activity of brostallicin with that of DDP. As can be seen from Table 5, whereas brostallicin showed a greater activity against GST-σ-overexpressing tumors (A2780/GST 8; TI >80%) than in tumors with normal levels of the enzyme (A2780/GST 16; TI = 36%), DDP showed a comparable activity maximum (TI = 45% and 48% for A2780/GST 8 and A2780/GST 16, respectively).

Experiments on the interaction of brostallicin with plasmid DNA (pUC18) were performed to verify a possible covalent binding between brostallicin and DNA. The covalent adducts were thermally unstable and spontaneously generated nicking of the double strand with subsequent relaxation of the plasmid supercoiled form, which was highlighted by a different electrophoretic band. Brostallicin alone did not react: a change of the DNA topology from supercoiled to the circular form (nicking) was observed when pUC18 was incubated with brostallicin, but only in the presence of the GSH/GST system (Fig. 4).

**DISCUSSION**

A new class of MGBs in which a potential alkylating moiety of low chemical reactivity was tethered to a distamycin A frame was generated by the synthesis of α-bromoacryloyl derivatives. Among these new compounds, brostallicin has been selected and is at present under Phase II clinical development.

The present study shows that brostallicin has a unique pharmacological profile, with its antitumor activity increased in tumors with high GSH/GST levels. Evidence has been reported based on different cellular models. Isogenic cell systems differing only for the expression of GST-σ isoenzyme allowed confirmation that the greater sensitivity to brostallicin occurs not only in vitro cultured cells, but also in tumors transplanted in nude mice. The absolute activity of brostallicin against cancer cells of different origin is not only related to the GST/GSH content, but other cellular factor are likely to account for its activity. The GST-catalyzed reaction of brostallicin with GSH increases its relative activity, and the difference is clearly observable in cells with similar genetic background and different GST/GSH content.

This interesting and unique feature is chemically plausible and involves the α-bromoacryloyl group of brostallicin, which in the presence of nucleophilic species, e.g., GSH, performs a first-step

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**Table 3** Results of incubation of brostallicin (10 μM) with GSH (1 mM) and GST enzymes or BSA (2.5 μg/ml)

<table>
<thead>
<tr>
<th>Incubation number</th>
<th>Incubation components</th>
<th>Mean (% consumed) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brostallicin</td>
<td>0.00 ± 2.11</td>
</tr>
<tr>
<td>2</td>
<td>Brostallicin + GSH</td>
<td>1.58 ± 7.67</td>
</tr>
<tr>
<td>3</td>
<td>Brostallicin + GSH + BSA</td>
<td>10.12 ± 4.96</td>
</tr>
<tr>
<td>4</td>
<td>Brostallicin + GSH + GST-P1-1</td>
<td>46.29 ± 5.66</td>
</tr>
<tr>
<td>5</td>
<td>Brostallicin + GST-A1-1</td>
<td>23.22 ± 13.51</td>
</tr>
<tr>
<td>6</td>
<td>Brostallicin + GST + GST-M1-1</td>
<td>49.78 ± 7.93</td>
</tr>
</tbody>
</table>

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**Table 4** Kinetics of incubation of brostallicin (1 μM) with GSH (1 mM) and GST enzymes (2.5 μg/ml)

<table>
<thead>
<tr>
<th>Incubation</th>
<th>t_{1/2} (min)</th>
<th>CL_{int} (ml/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brostallicin + GSH + GST-P1-1</td>
<td>0.82</td>
<td>337.0</td>
</tr>
<tr>
<td>Brostallicin + GSH + GST-A1-1</td>
<td>58.35</td>
<td>4.8</td>
</tr>
<tr>
<td>Brostallicin + GSH + GST-M1-1</td>
<td>0.43</td>
<td>642.6</td>
</tr>
<tr>
<td>Brostallicin + GSH</td>
<td>41.24</td>
<td>7.93</td>
</tr>
</tbody>
</table>

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**Fig. 2. In vitro brostallicin activity in isogenic systems differing in the expression of GST-σ isoenzyme.** A, A2780-derived clones transfected with human GST-σ-cDNA and presenting different GST activities. □ A2780/GST 7; ○ A2780/GST 8; ● A2780/GST 16. B, growth inhibition induced by different brostallicin concentrations in parental and GST-σ-transfected MCF-7 cells. ● MCF-7/new; ○ MCF-7/pmTG-5. Bars, SD.
Michael-type attack, which may be followed by a further reaction of the no longer vinlyc halogen, leading to alklylation of nucleophilic functions such as those present in the DNA. The reaction between brostallicin and GSH is catalyzed by GST, with the $\pi$ and $\mu$ isoenzymes being more effective than the $\alpha$ isoenzyme.

This might be important clinically because GSH and GST overexpression in comparison with normal tissues occurs de novo in several cancers because GST-$\pi$ is the most prevalent GST isoenzyme in tumors (23, 33, 34). Preclinical and clinical studies have established an association between GSH/GST overexpression and cancer, and several studies have been performed to determine whether their levels have prognostic significance. GSH/GST overexpression develops in a considerable proportion of tumors in association with acquired resistance to many DNA-damaging agents and has been correlated with a poor prognosis (22, 23, 28, 35, 36).

Furthermore, in different experimental cellular systems in vitro, treatment with antitumor drugs, such as classical alkylating agents, platinum derivatives, and anthracyclines, induces overexpression of GST (22, 23, 28, 35–37–39). This suggests that brostallicin may be used as an alternative to or in combination with other drugs. This interesting opportunity is presently under investigation in clinical trials.

In conclusion, brostallicin represents a novel cytotoxic antitumor compound whose therapeutic index in preclinical models is significantly improved in comparison with other MGBs, still retaining the significant efficacy in a broad spectrum of preclinical tumor models that characterized the earlier MGBs. Importantly, brostallicin activity is increased, at least in defined isogenic models, by the GST content. If confirmed in clinical studies, this could represent a major advantage because many drugs lose their activity in tumors with high GSH/GST content. During clinical studies, the activity of the drug will be correlated, whenever possible, with the tumor GST content, although it is to be expected that a significant correlation can be observed only with many patients. Considering that human tumors show at least equal, but often increased GST/GSH expression compared with normal tissues (23, 26, 27), the compound offers the unique advantage of potentially having a higher therapeutic window and efficacy in tumors that are refractory to classical anticancer agents.

REFERENCES

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