

Antitumor activity of brostallicin on human prostatic cancer: role of combination with hypomethylating agents

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M. Broggini¹, M.A. Sabatino¹, C. Geroni², S.W. Weitman³

1. Istituto Mario Negri, Milan, Italy. 2. Nerviano Medical Sciences, Oncology, Nerviano, Italy. 3. Systems Medicine LLC, a wholly owned subsidiary of Cell Therapeutics Inc., Seattle, WA USA.

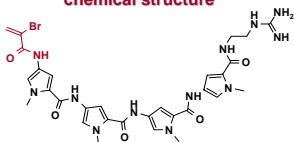


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Background

● Brostallicin (**Fig.1**) is a DNA minor groove binder, currently in Phase I/II trials as a single agent or in combination.

Fig. 1 - BROSTALLICIN chemical structure



α-bromoacrylic derivative with a distamycin-like structure composed of four pyrrolocarbamoyl units in series and ending with a guanidine moiety.

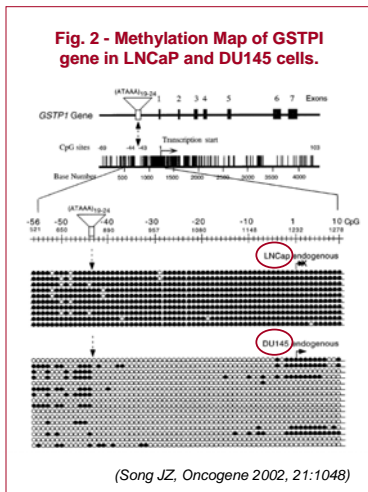
● Brostallicin preclinical antitumor activity depends on the intracellular levels of glutathione (GSH) and/or glutathione S-transferase (GST). Among the GST isoenzymes the pi class is its stronger activator. The use of isogenic cell systems differing only for the expression of the GST-pi isoenzyme, verified that an increase in antitumor activity of brostallicin is related to higher levels of GSTpi activity, not only in *in vitro* cultured cells, but also in tumors transplanted in nude mice. (Geroni C, 2002; Beria I, 2004; Broggin M, 2004).

● Molecular characterization of prostate cancer has shown that about 90% of human prostatic tumors carry a strong methylation of the GSTP1 promoter gene. This epigenetic modification accounts for the absence of GSTpi enzyme, starting from the early tumorigenesis. Therefore, GSTP1 hypermethylation assays are being developed for prostate cancer detection. (Nakayama M, 2004; Esteller M, 1999; Harden SV, 2003).

● This epigenetic modification could be reverted and GST-pi enzyme re-expressed using compounds inhibiting DNA cytosine methylation (hypomethylating drugs). The most characterized and widely used drug is the nucleoside analog, 5-aza-2'-deoxy-cytidine, but due to its toxicity-duplicate phase, other hypomethylating approaches have been developed such as the use of the nucleoside analog zebularine and combination with histone acetylation inhibitors. (Issa JPJ, 2007; Cheng JC, 2004).

● The human prostatic carcinoma cell line LNCaP retains this molecular characteristic (**Fig. 2**) with very low GST enzymatic activity (**Fig. 3A**) (Song JZ, 2002). Conversely, the human prostatic carcinoma cell line DU145 presents a low level of promoter methylation (**Fig 2**) and high GST enzymatic activity.

● We used these cell lines to evaluate in *in vitro* and *in vivo* studies, the effect of hypomethylating drugs/brostallicin combination therapy.



Aim of the study

● To test the activity of brostallicin on human prostatic carcinoma models with different levels of GSTP1 promoter methylation. Two cell lines were tested: LNCaP cells with very high GSTPI methylation and DU145 cells with low GSTPI methylation.

● To verify whether LNCaP cells can be sensitized to brostallicin activity by modulating GST-pi expression (LNCaP clones transfected with human GSTpi cDNA were isolated and tested).

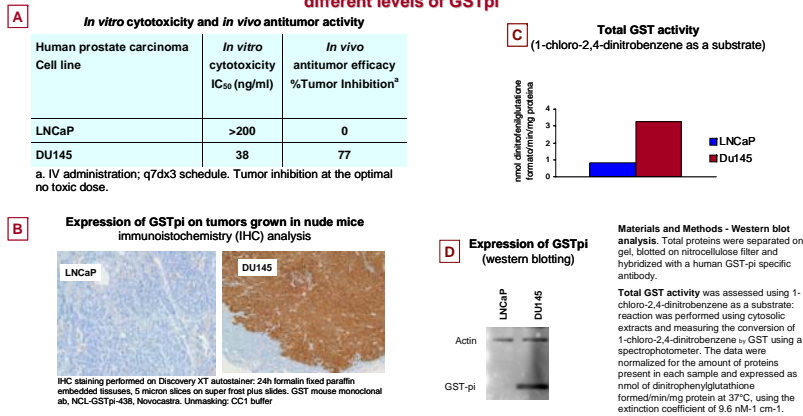
● Since DNA methylation is an epigenetic modification reversible through DNA methyltransferase inhibitors, the antitumor effect of brostallicin was tested *in vitro* and *in vivo* on LNCaP cells pre-treated with the hypomethylating agent zebularine.

● **Fig. 3** Reports the activity of brostallicin on LNCaP and DU145 human prostatic carcinoma models with very high or low methylation levels in GSTP1 promoter, respectively.

● **Brostallicin** showed *in vitro* and *in vivo* differential activity against the two cell lines (**A**) being **more effective on DU145 cells** (IC₅₀ 38 ng/ml and 77% tumor inhibition) **than on LNCaP cells** (IC₅₀ >200ng/ml and no efficacy in SCID mice). In addition, GSTpi expression in xenograft tumors by IHC (**B**) showed strong GSTpi positivity only in DU145 tumor cells.

● **GSTpi expression correlates with the sensitivity to brostallicin** as shown by the GST total enzymatic activity (**C**) that is higher in DU145 cells compared with LNCaP cells (3.2 nmol and 0.8 nmol dinitrophenylglutathione formed/min/mg proteins in cytosolic extracts, respectively and GSTpi protein expression (**D**) which was detectable only in DU145 cells.

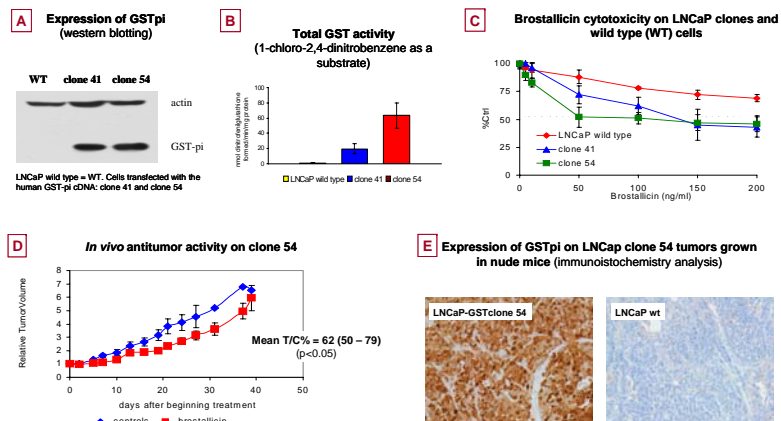
Fig. 3 - In vitro and in vivo activity of brostallicin on human prostatic carcinoma cell lines with different levels of GSTpi



● To verify whether LNCaP cells can be sensitized to brostallicin activity by modulating GST-pi expression, **LNCaP cells were transfected with the human GST-pi cDNA**. Two clones (clone 41 and clone 54) were selected and tested in comparison with LNCaP wild type cells (**Fig. 4**).

● **Clones showed higher GST-pi protein (A) expression and GST total enzymatic activity (B) compared to parental LNCaP cells.** *In vitro* and *in vivo* experiments showed that **brostallicin was more cytotoxic on clones** (IC₅₀ ~50ng/ml on both) **than on wild type LNCaP** (IC₅₀ >200 ng/ml) cells (**C**) and it was **effective in vivo only on GSTpi cDNA transfected LNCaP** clone 54 (**D**). **Strong GSTpi staining positivity** was also detected by IHC in LNCaP cells from clone 54 (**E**).

Fig. 4 - In vitro and in vivo activity of brostallicin on isogenic human prostatic carcinoma LNCaP cell lines



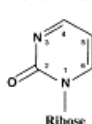
Materials and Methods - LNCaP cells were transfected with the human GSTpi cDNA, subcloned in the pcDNA3 vector, using gene porter transfection system. After transfection, clones were selected in medium containing the selection antibiotic (G418). Two clones, clone 41 and 54 were selected for further analysis. Cytotoxicity evaluated by MTT assay, 72h treatment LNCaP-GST clone 54, transplanted in SCID mice was treated with brostallicin (2) (0.4 mg/kg, q14dx2) Controls were treated with saline (2). The relative tumor volume (RTV) was plotted against time (days). RTV values of treated group resulted significantly (ANOVA test) reduced vs control group (p<0.05) starting 2 days after the last treatment with brostallicin.

Results

- DNA methylation is an epigenetic modification reversible through DNA methyltransferase inhibitors. Zebularine is a cytidine analog containing a 2-(1H)-pyrimidinone ring that was developed as a cytidine deaminase inhibitor. The molecule is an inhibitor of DNA methylation which is stable and minimally toxic both *in vitro* and *in vivo*. (Cheng JC, 2003, 2004).

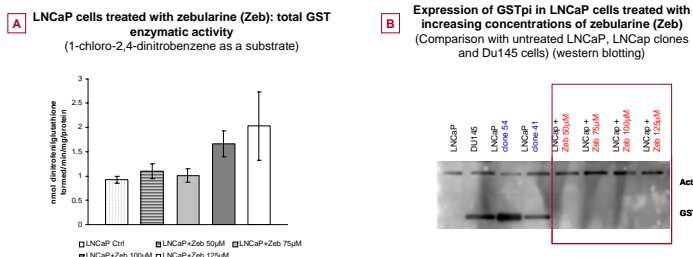
- **Fig. 5** Reports data obtained in LNCaP cells treated *in vitro* with zebularine (100-125 μ M) for 96h. **Cells treated with zebularine showed increased intracellular level of GST enzymatic activity** compared to untreated ones (A). Conversely, by Western Blotting assay, **no detectable expression of GSTpi protein was found** at any of the tested concentrations (B).

Zebularine



1-(β -D-ribofuranosyl)-1,2-dihydropyrimidin-2-one

Fig. 5 - LNCaP cells treated *in vitro* with zebularine

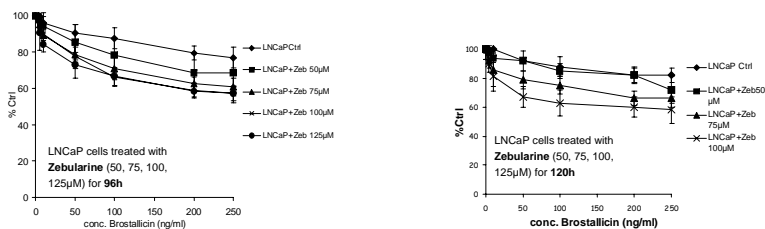


Materials and Methods - Total GST activity was assessed using 1-chloro-2,4-dinitrobenzene as a substrate: reaction was performed using cytosolic extracts and measuring the conversion of 1-chloro-2,4-dinitrobenzene to GST using a spectrophotometer. The data were normalized for the amount of proteins present in each sample and expressed as nmol of dinitrophenylglutathione formed/min/mg protein at 37°C, using the extinction coefficient of 9.6 nm⁻¹ cm⁻¹. Data are the mean of three separate experiments.

Western blot analysis. Total proteins were separated on gel, blotted on nitrocellulose filter and hybridized with a human GST-pi specific antibody.

- The **cytotoxicity of brostallicin** was evaluated on LNCaP cells pretreated with **zebularine**. Cells were treated with different doses of zebularine (50, 75, 100, 125 μ M) for 96 or 120 h before the co-treatment with brostallicin for 72 h.
- Dose response curves (Fig. 6) showed that the LNCaP cells, pretreated with zebularine, were more responsive to brostallicin than the untreated ones. The effect was dose-dependent.

Fig. 6 - *In vitro* cytotoxicity of brostallicin on LNCaP cells treated with zebularine (Zeb) for 96 or 120h before the co-treatment with brostallicin (72h)



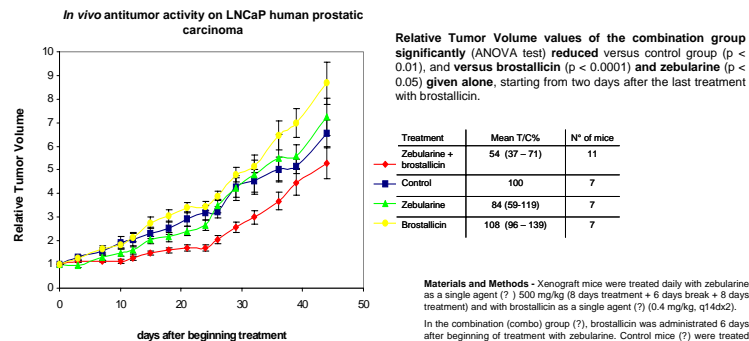
Materials and Methods - Brostallicin was dissolved as stock solution in DMSO 20% and PBS; zebularine was dissolved in PBS. Human prostatic cancer cells LNCaP were maintained in RPMI medium, 10% FCS. Cells were treated for 96-120 h with zebularine and then treated with increasing concentrations of brostallicin. Cells were treated with brostallicin continuously for 72h. (MTT assay). The cytotoxic effect of combination brostallicin - zebularine was significantly (ANOVA test) higher versus control group ($p < 0.01$ - $p < 0.003$ for time 96h zebularine 75, 100 and 125 μ M and $p < 0.05$ - < 0.005 , for time 120h zebularine 75 and 100 μ M)

- *In vivo* studies are reported in Fig. 7. LNCaP cells were transplanted subcutaneously in SCID mice. When the tumor was palpable (0.2-0.3 g), animals were treated daily x6 with zebularine (IP, 500 mg/kg) and on day 6 were treated with brostallicin (IV, 0.4 mg/kg). The schedule was repeated twice.
- GSTpi protein expression was evaluated in LNCaP tumors treated with zebularine. Tumor fragments were excised from sacrificed mice the day after the last administration of zebularine and both Western Blotting on protein extracts and IHC for GSTpi expression were performed. **The treatment with zebularine did not induced detectable amounts of GSTpi protein expression** in LNCaP tumors and **no evidence of GSTpi expression** was found in samples examined by IHC (data not shown).

- As regards the efficacy, **brostallicin and zebularine as single agent, at the maximum tolerated doses did not show any antitumor effect on LNCaP human prostatic carcinoma model** as expressed by the mean T/C% values of 108 and 84, respectively.

- On the contrary, the **co-administration of brostallicin and zebularine showed a mean T/C% value of 54%**, thus supporting a possible **role of zebularine in increasing the antitumor activity of brostallicin in a tumor with no expression of GSTpi due to GSTP1 promoter hypermethylation**.

Fig. 7 - LNCaP cells treated *in vivo* with zebularine



Conclusions

- The CpG-rich promoter of the GSTP1 gene (encoding for GST-pi enzyme) is hypermethylated in the majority (70-95%) of human prostatic adenocarcinomas. DNA methylation is an epigenetic modification reversible through DNA methyltransferase inhibitors.
- The present work describes a new approach for the treatment of prostate cancer based on the use of the hypomethylating agent zebularine in combination with brostallicin whose efficacy is increased in tumors with higher GSTpi levels.
- Brostallicin shows activity against the human prostatic carcinoma model DU145 which expresses GST-pi, while is inactive against LNCaP tumor that does not express GST-pi protein due to heavy methylation of GSTpi gene promoter. Re-introduction of the human GST-pi cDNA in LNCaP cells restores sensitivity to brostallicin treatment.
- The data so far obtained shows that LNCaP prostate carcinoma cells with heavily methylated GSTP1 gene can be rendered more sensitive to brostallicin after treatment with zebularine. However, qualitative methods of investigation, such as Western Blotting and immunohistochemistry do not detect any expression of GSTpi protein.
- Additional studies are ongoing aimed at evaluating whether quantitative methods of methylation analysis, such as pyrosequencing, are able to appreciate differences in the methylation degree after treatment with zebularine.
- Moreover, this model need to be further investigated in order to elucidate how and when a sustained demethylation of various loci is reachable and how to prevent a possible remethylation.