

M. Ciomei¹, C. Albanese¹, P. Della Torre¹, A.M. Giusti¹, N. Mongelli¹, S. Weitman², K. Shanmugam², E. Bruckheimer², C. Geroni¹

1. Nerviano Medical Sciences, Nerviano, Italy; 2. System Medicine LLC, Tucson, AZ

Background

Brostallicin (Fig. 1) is a DNA minor groove binding drug under evaluation in Phase I and II clinical trials.

One of the unique mechanisms of action for brostallicin is its ability to interact covalently with DNA in the presence of glutathione and glutathione S-transferase (GSH/GST) (Geroni C. 2002; Berra I. 2004; Brogioni M. 2004), a detoxifying system responsible for resistance to common anticancer agents. As a consequence, brostallicin has enhanced activity in cancer cells with high GSH/GST levels.

Previous data demonstrated that brostallicin strongly induced apoptosis in tumor cells and the levels of GSH correlate with its cytotoxic and apoptotic potential.

The tumor suppressor p53 acts as a major defense against cancer (Yael A. 2007; Vousden KH. 2007). Mutations in p53 are a hallmark of at least 50% of all human cancers (Soussi T. 2005). Since p53 plays a central role in apoptotic signaling and cell cycle arrest from DNA damaging agents, we tested the *in vitro* effects of brostallicin on cell viability, cell cycle modulation and apoptosis induction in cell lines expressing either functional or altered p53 status.

Aim of the Study

The objective of this study was to evaluate whether p53 status plays a role on the activity of brostallicin against cancer cells.

The effects of brostallicin on cell viability, cell cycle modulation and apoptosis induction were tested on cell lines expressing either functional p53 (A2780 - human ovarian carcinoma and HCT116 - human colon carcinoma) or altered p53 (HCT116 E6 - p53 negative and HT29 - p53 mutated human colon carcinomas).

In addition, a correlation between cell growth inhibition and p53 status was made testing brostallicin on 80 different tumor cell lines.

Conclusions

Brostallicin treatment generated a pronounced cell cycle block (S-G2 phase) and a significant induction of apoptosis in all tumor cell lines tested regardless of the p53 status.

Although the sensitivity of p53 wild type cells seems in some cases higher than that of p53 null or mutated cell, when brostallicin cytotoxic effect was evaluated on a large number of cell lines, no statistically significant correlation was found between the p53 status and cell growth inhibition.

These results will help guide the clinical development of brostallicin and suggest that cancer patients may benefit from treatment regardless of the p53 status.

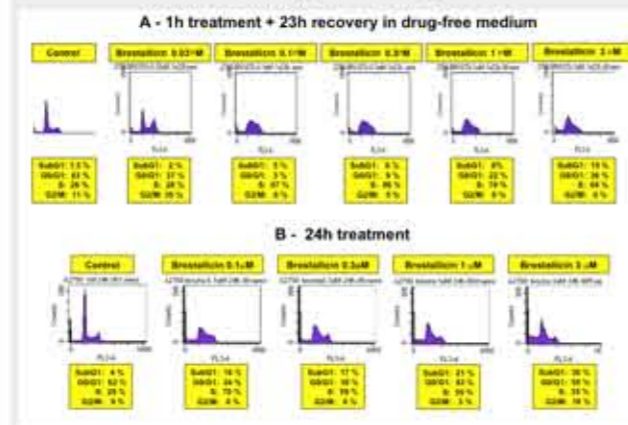
1. Brostallicin Inhibits Cancer Cell Proliferation Through the Cell Cycle Arrest and Induction of Apoptosis – *in Vitro* and *in Vivo* Studies on A2780 Human Ovarian Carcinoma Cells

A2780 human ovarian carcinoma cell line harboring the wild-type p53 gene and expressing measurable levels of GSH and GST have been used to investigate the *in vitro* and *in vivo* ability of brostallicin to inhibit tumor growth.

Doses were chosen on the basis of the 50% inhibitory concentration (IC₅₀) obtained after 1h (IC₅₀ = 0.018 μM) and 24h (IC₅₀ = 0.003 μM) exposure to brostallicin in the proliferation assay at 72h.

The effect of brostallicin on cell cycle modulation was tested after either 1h treatment + 23h recovery in drug-free medium or 24h continuous treatment (Fig. 2).

Fig. 2 - Cell Cycle Perturbation Induced by Brostallicin on A2780 Cells After 1h (A) and 24h (B) Treatment



Clear cell cycle perturbation was already present after 1h treatment at the lower dose of brostallicin with an accumulation of cells in G2 phase and, increasing the dose, a complete block in S phase (Fig. 2 A).

With prolonged time of treatment (Fig. 2 B) the block in S phase persisted and an increase in subG1 (cells with fragmented DNA) became evident.

The parallel evaluation of mitotic index showed a decrease and, at higher doses, a disappearance of mitotic events in cells treated with brostallicin (Figure 3).

Fig. 3 – Evaluation of Mitotic Index in A2780 Cells Exposed to Brostallicin



Cells were treated with different concentrations of brostallicin for 1h + 23h release in drug-free medium or 24h.

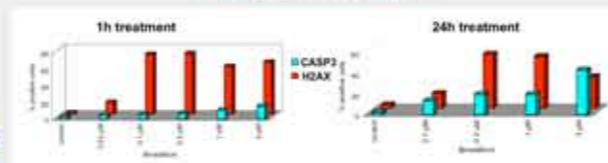
Phosphorylation on Ser 10 of histone H3 (pH3) is associated with chromatin condensation that occurs during mitosis so the % of pH3 is considered as mitotic index.

A zeroing of the mitotic events in treated cells is evident at all the tested doses.

Fig. 4 summarizes the effect of brostallicin in inducing apoptosis using both caspase-3 activation and phosphorylation of histone H2AX, which are considered the earliest indicators of apoptosis as they occur long before the evidence of morphological changes and the increase in subG1 (as shown in Table 1).

Brostallicin showed a dose-dependent activation of caspase-3 and a significant increase in H2AX.

Fig. 4 – Evaluation of Apoptosis Induction in A2780 Cells Exposed to Brostallicin



Cells were treated with different concentrations of brostallicin for 1h + 23h release in drug-free medium or 24h.

Casp3 and H2AX = Caspase-3 activation and phosphorylation of histone H2AX on serine 139 were used to evaluate apoptosis induction in treated versus untreated cells.

Table 1 – Determination of subG1 Population in A2780 Cells Treated with Brostallicin

Treatment	Dose (μM)	cells in subG1	
		1h	24h
Control	0	1.5%	4%
Brostallicin	0.03	2%	-
	0.1	5%	16%
	0.3	6%	17%
	1	8%	21%
	3	15%	26%

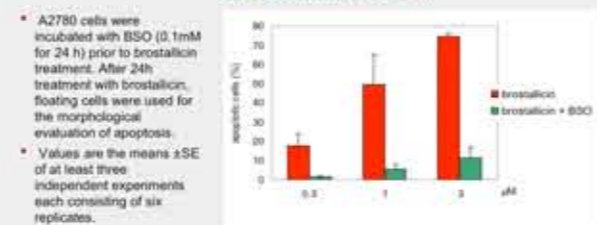
Percentage of cells with a content of DNA lower than that of G1 phase (fragmented DNA) indicating the presence of apoptosis and necrosis.

There is a time and dose-dependent increase in subG1 which is clearly evident at the higher doses of brostallicin and with 24h treatment.

Since previous data showed that GSH plays a relevant role in activating and increasing the antitumor activity of brostallicin, we tested the pro-apoptotic activity of brostallicin in cells pretreated with the GSH-inhibitor BSO.

In Fig. 5, depletion of GSH decreased the apoptotic effect of brostallicin (11.5% of apoptotic cells in BSO-pretreated cells vs 74.5% in BSO-untreated cells at the higher tested dose of 3μM) supporting its role in brostallicin activity.

Fig. 5 – Apoptotic Effect of Brostallicin on A2780 Cells Pretreated with the GSH-inhibitor DL-buthionine-[S,R] sulphoximine (BSO)



We next investigated the correlation between the *in vivo* antitumor activity of brostallicin and its effect on cell cycle or on apoptosis induction on subcutaneously implanted A2780 xenografted tumors.

A single i.v. administration of brostallicin showed 59, 69, 79 and 91% tumor inhibition one week after treatment at the tolerated doses of 0.52, 0.8, 1.2 and 1.8 mg/kg, respectively (Fig. 6).

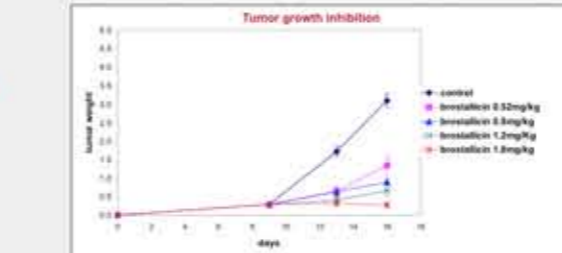
Fig. 6 – *In Vivo* Anti-Tumor Activity of Brostallicin in the A2780 Human Ovarian Xenograft Model

	Dose (mg/kg)	T1% ^a	TOX ^b	Maximal Body Weight reduction % (day)
Brostallicin	0.52	59	0/7	0
	0.80	69	0/7	8 (13)
	1.20	79	0/7	4 (13)
	1.80	91	0/7	11 (16)

Tumor fragments were implanted subcutaneously. Treatment started when tumors were about 300 mg. Brostallicin was administered intravenously; single treatment.

a. T1%: Inhibition of tumor growth 1 week after the last treatment.

b. TOX: Number mice died for toxicity/total number mice.



Tumors were excised 24h after brostallicin treatment, cell cycle analyzed by flow cytometry and the number of apoptotic and mitotic cells evaluated microscopically.

Cell cycle analysis revealed a clear accumulation in G2/S phase at all the tested doses confirming the "in vitro" results.

The amount of apoptotic cells (Table 2) was 3.3-6 times higher in brostallicin treated tumors treated vs. untreated tumors (p<0.01).

Brostallicin showed a 3.8 – 23 fold decreased mitotic cells compared to that of controls (p<0.01).

A clear dose-response was observed for the parameters evaluated *in vivo* (apoptotic index, mitotic index) as well as antitumor efficacy.

Table 2. Apoptotic and Mitotic index in A2780 Tumors 24h Following i.v. Treatment with Brostallicin

		Brostallicin				
		0.52 (mg/kg)	0.8 (mg/kg)	1.2 (mg/kg)	1.8 (mg/kg)	
Apoptosis	Cell number (mean ± S.D.)	3.5 ± 0.4	13.07 ± 1.3**	11.6 ± 3**	13.3 ± 2.8**	22.03 ± 4.6**
	AI	0.75	2.8	2.5	2.9	4.73
Mitosis	Cell number (mean ± S.D.)	1.07 ± 0.1	0.27 ± 0.06*	0.10 ± 0.03**	0.03 ± 0.01**	0.07 ± 0.02**
	MI	0.23	0.06	0.02	0.01	0.01

AI = Apoptotic index: percentage of the mean value of cells with apoptotic morphologies per microscopic field of a mean of total number of cells per microscopic field (465 cells).

MI = Mitotic index: percentage of the mean value of cells with mitotic morphologies per microscopic field of a mean of total number of cells per microscopic field (465 cells).

*p<0.05; **p<0.01

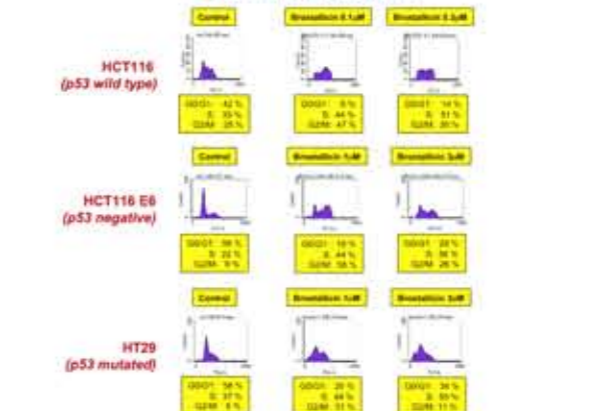
2. Brostallicin Effect on Cell Cycle Modulation and Apoptosis Induction in Cancer Cells Differently Expressing p53

The activity of brostallicin was tested on tumor cell lines differently expressing p53, an important determinant for the responsiveness to treatment with DNA-interacting agents.

The following human colon carcinoma cell lines were used: HCT116 (p53 wild type), HCT116E6 (p53 negative) and HT-29 (p53 mutated). Cells were treated with different concentrations of brostallicin. Doses were chosen on the basis of the IC₅₀ previously obtained in the proliferation assay after 72h continuous treatment (IC₅₀ HCT116 = 0.032 μM, IC₅₀ HT29 = 0.157 μM, IC₅₀ HCT116 E6 = 0.211 μM).

For all the three cell lines tested, the effect of brostallicin on cell cycle modulation is particularly strong after 24h of continuous treatment with a clear and massive accumulation in G2-S phase of the cell cycle, induced already at the lower dose (0.1 μM for HCT116 and 1 μM for both HT29 and HCT116E6 cells) (Fig. 7).

Fig. 7 – DNA Histograms of Colon Cancer Cells Treated for 24h with Brostallicin



In addition, the cytotoxic effect of brostallicin on 80 different cancer cell lines (23 cell lines with p53 wild type; 53 cell lines with p53 mutated and 4 cell lines with p53 negative) was correlated to p53 status.

The average IC₅₀ were 0.321, 0.468 and 0.456 μM on p53 wt, p53 mutated and p53 negative cells, respectively. As shown in Fig. 10, the cytotoxic effect of brostallicin was not significantly different on cancer cells with wild type p53 versus p53 mutated or null cells (p = 0.31).

Fig. 9 – Correlation Between Brostallicin Cytotoxicity and p53 Status on 80 Tumor Cell Lines (72h treatment)

