

Abstract

Cytorex® is a novel anticancer drug candidate which uses Hydrofluoric and Sulfuric acids as active compounds. The Institute of Drug Development evaluated the compound for the sponsor, Cytorex Biosciences, Inc., in an in vivo murine model of B16-F10 melanoma lung metastases to gain insight into the mechanism of drug action. Upon completion of an in vivo study performed under a separate agreement, formalin preserved lung tissue with B16-F10 metastases was provided for biomarker analysis. Quantitative analysis of Bcl-2, Bax, Smac/Diablo, Survivin, Raf-1, and (P) Raf-1 was performed in thin sections from three representative tumor metastases selected from the drug treatment group (.654 mg/mouse, p.o., qd x 5 to end) and the corresponding control vehicle. Two biomarkers (Bcl-2, Smac/Diablo) were below detection limits. Cytorex® induced some down regulation in Bax. Among the evaluable biomarkers expression levels of Survivin and the (P) Raf-1/Raf-1 ratios were decreased in the Cytorex® treatment group. These findings are consistent with the pro-apoptotic activity of the drug and the ability of Cytorex® to interfere with tumor signaling pathways in B16-F10 lung metastases.

Cytorex®

Is a novel drug that is being developed by Cytorex Biosciences, Inc. (Cytorex) (www.cytorex.com). Cytorex® (further referred to as Cytorex) is an acidic transparent liquid with appearance similar to water that is innocuous to the skin on contact, and uses inorganic acids as active compounds. The drug has cytotoxic, selective apoptotic, antiviral and antineoplastic properties. It does not change concentration with time and is not photosensitive. Cytorex is isotonic in blood, and can be administered orally and by IV. Cytorex has demonstrated potent activity in vitro and in vivo. These studies are being expanded into in vivo animal models to validate drug biomarkers and mechanism of action.

Study Objective

The specific aim of this small pilot study was to determine the effect of Cytorex on expression of select biomarkers in B16-F10 murine melanoma lung metastases to gain insight into the mechanism of drug action. Quantitative analysis of biomarkers (Bcl-2, Bax, Smac/Diablo, Survivin, Raf-1 and (P)Raf-1), was performed.

Materials And Methods

Animal Tissue

Murine lung tissue with B16-F10 melanoma metastases was harvested by the Preclinical Research Laboratory and preserved in buffered formalin. Following the quantitation of B16-F10 lung metastases that was performed under a separate agreement and budget, the tissue was transferred to the Molecular Targets Laboratory for further processing.

Biomarker Analysis

The tissue was dehydrated through graded ethanol and embedded in paraffin. Thin sections were then prepared from paraffin blocks. Slides were deparaffinized and stained with anti-murine antibodies as follows: Bcl-2 (Neo Markers, Fremont, CA), Bax (Cell Signaling, Beverly, MA), Smac/Diablo (Santa Cruz Biotechnology, Santa Cruz, CA), Survivin (Novus, Littleton, CO), Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA), (P) Raf-1 (serine 259) (Cell Signaling, Beverly, MA). Quantitative analysis of biomarkers was performed in thin sections from three representative tumor metastases selected from the drug-treatment group (Cytorex at 0.654 mg/mouse p.o, qd x 5 to end) and the corresponding control (vehicle p.o, qd x 5 to end) – Table 1 - by immunostaining with murine-specific antibodies.

Table 1
Cytorex® vs. B16-F10 Murine Metastatic Melanoma Model

Group	n	Dose (mg/mse)	Route & Schedule	Wt.Change (Day 5)	Wt. Change (Day 15)	Mean Lung Wt(mg)±SD	Mean Met #±SEM	MGI(%)	# of Death	P-values
Control ¹	-10	---	I.V.; qd x 5 to end	+ 6.2%	+ 12.7%	466.1±186.7	140.5±23.9	---	0	---
Control ¹	-10	---	p.o.; qd x 5 to end	+ 4.1%	11.50%	231.0±12.4	78.5±9.6	---	0	---
CYTOREG® ²	-10	0.145	I.V.; qd x 5 to end	+ 6.2%	11.70%	272.1±11.0	60.2±8.8	57.2	1	0.0077
CYTOREG® ²	-10	0.218	I.V.; qd x 5 to end	+ 8.7%	16.90%	286.7±23.3	51.3±9.9	63.5	0	0.0028
CYTOREG® ²	-10	0.654	p.o.; qd x 5 to end	+ 3.8%	+ 9.5%	291.7±27.3	39.1±5.2	50.2	0	0.0020
Paclitaxel ³	-10	16mg/Kg	I.p.; qd x 5	- 6.9%	+ 5.9%	190.8 ± 9.8	34.6 ± 4.5	75.4	0	0.0006

Antigens were detected after incubation with appropriate secondary antibodies followed by DAB staining and methyl green counterstaining. The expression of biomarkers was determined using a scoring system of Allred et al (1), whereby immunostaining signals are assigned a proportion score (PS, range 0-5) that represents the estimated proportion of positive tumor cells on the entire slide and the intensity score (IS) that estimates the average staining intensity of positive tumor cells (range 0-3). The PS and IS are added to obtain a total score (TS, range 0-8). The numbers of cells and their TS were determined.

Adjacent sections were stained with hematoxylin and eosin (H&E) for assessment of morphology. Appropriate positive control (thymus) and negative controls were included. Processing and staining of tumor tissue was performed at the Pathology Core at the University of Texas Health Science Center at San Antonio.

Results And Discussion

In vivo experiments conducted by the Preclinical Research Laboratory at the CTCRC Institute for Drug Development have shown that Cytorex reduced the number of lung metastases in the B16-F10 murine melanoma model. The goal of this pilot study was to evaluate the effects of the drug on select biomarkers representing diverse pathways such as apoptosis (Bcl-2, Bax, Smac/Diablo), signal transduction (Raf-1, P-Raf-1), and survival (Survivin). Based on the in vivo results (Table 2), representative tumor metastases were selected in the treatment groups corresponding to the control (group 2; vehicle p.o, qd x 5 to end) and the highest dose of Cytorex (group 5; Cytorex at 0.654 mg/mouse p.o, qd x 5 to end). Notably, this dose of Cytorex reduced the number of lung metastases by >50%. For a preliminary assessment of drug effects on biomarker expression, we have selected three representative specimens in the control and the drug treatment group, based on the numbers of metastases closest to the means in each group.

The expression of Bcl-2, Bax, Survivin, Smac/Diablo, Raf-1 and its activated form (P) Raf-1, was quantitatively assessed in the lung metastases. A summary of the immunohistochemical data is shown in Table 2, along with the total scores for the examined biomarkers.

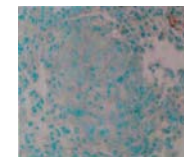
Table 2
Effects Cytorex® on Biomarker status in B16-F10 Melanoma Lung Metastases

Animal ID	Bcl-2	Bax	Survivin	Raf-1	(P) Raf-1	Smac
#2 0.654mg	0	1+	2+	*1+	2+	0
#6 0.654mg	0	2+	1+	0	1+	0
#8 0.654mg	0	3+	2+	1+	2+	0
Total Cytorex	0	6	5	2	5	0
#3 0.654mg	0	3+	3+	1+	2+	0
#2 0.654mg	0	3+	2+	0	2+	0
#2 0.654mg	0	3+	2+	1+	2+	0
Total Cytorex	0	9	7	2	6	0

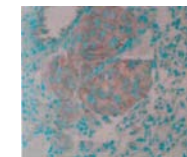
* cytoplasm and nucleoli

Bcl-2 and Bax/Bcl-2

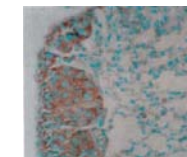
Bcl-2 and Bax/Bcl-2 were undetectable in the Cytorex and the control groups. The expression of the pro-apoptotic Bax was somewhat decreased the lung metastases in the Cytorex treatment group. However, the consequences of that event on apoptosis of tumor cells may not be significant, considering very low Bcl-2 levels, as the Bcl-2/Bax ratio is a stronger indicator of the cell commitment to apoptosis.



Bcl-2 (Control)



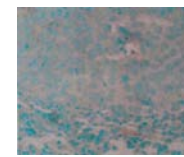
Bax (Cytorex®)



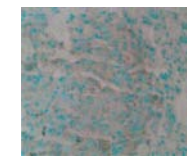
Bax (Cytorex®)

Raf-1 and P-Raf-1

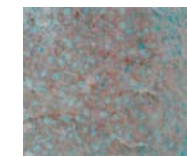
Raf-1 levels were unchanged in the control and Cytorex treatment group, but in the latter, some Raf-1 was found partitioned between the cytoplasm and the nucleoli. The phosphorylated Raf-1 decreased from a total score of 6 (control) to a score of 5 (Cytorex). Taken together with somewhat decreased cytoplasmic localization and phosphorylation of Raf-1, Cytorex might have an effect on the Raf-1 activation and regulation of the Raf-1 signaling pathway.



Raf1 (Cytorex®)



p-Raf1 (Cytorex®)



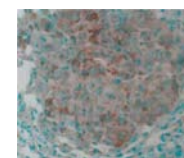
p-Raf1 (Cytorex®)

Smac/Diablo

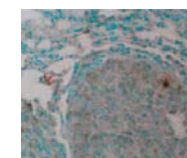
Smac/Diablo was undetectable in the specimens, although the antibody cross-reacted with a positive control tissue.

Survivin

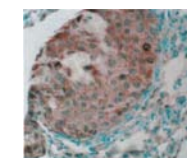
Images of Survivin in lung metastases and the control tissue are shown in picture. The SURVIVIN Score decreased from a total of 7 in the vehicle control to a total of 5 in the Cytorex treatment group (see Table 2), suggesting a anti-apoptotic activity of the drug.



Survivin (Cytorex®)



Survivin (Cytorex®)



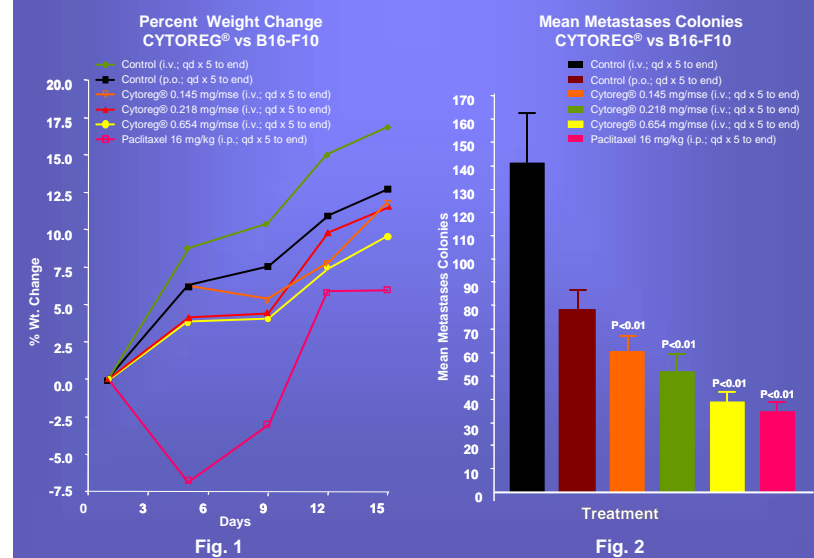
Survivin (Control)

Summary and Conclusions

We have examined the expression of six tumor biomarkers in B16-F10 lung metastases from 3 vehicle controls and 3 Cytorex-treated animals. Two biomarkers (Bcl-2, Smac/Diablo) were not detected in the metastases, although the signal could be measured in the positive control tissue. Among the evaluable biomarkers, Survivin levels and the (P) Raf-1/Raf-1 ratios were apparently lower in the Cytorex group, suggesting a potential pro-apoptotic activity of Cytorex and the ability of the drug to interfere with tumor signaling pathways. The results of this pilot study warrant more extensive evaluation of Cytorex on expression of Survivin and activated Raf-1. Validation of the data in a larger study might yield relevant biomarkers for the clinical trials with Cytorex.

It is Important to emphasize that at the dose level and schedules evaluated, animals experienced little toxicity with Cytorex in the study, as only one mouse in low dose group died in the study. Cytorex at the 0.218 mg/mouse dose level achieved the best metastatic growth inhibition, with an MGI of 63.5% compared to the i.v. vehicle control (p = 0.0028). In comparison, the Paclitaxel group showed a 75.4% MGI. However, no weight loss was seen in the Cytorex groups compared to Paclitaxel. (Fig.1 and Fig. 2)

Murine Metastatic Melanoma Model



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For further information