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ORIGINAL ARTICLE

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Synergistic cytotoxicity of the ribonucleotide reductase inhibitor didox (3,4-dihydroxy-benzohydroxamic acid) and the alkylating agent carmustine (BCNU) in 9L rat gliosarcoma cells and DAOY human medulloblastoma cells

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Abstract *Purpose*: Ribonucleotide reductase (RR) is the rate-limiting enzyme of de novo DNA synthesis and has been shown to be upregulated linked with proliferation and malignant transformation. It was therefore identified as an excellent target for antitumor therapy. In the present study we investigated the biochemical and cytotoxic effects of didox, an inhibitor of RR, as a single agent and in combination with BCNU, an alkylating anticancer drug, in 9L rat gliosarcoma cells and DAOY human medulloblastoma cells. *Methods*: The effect of didox on the intracellular concentrations of deoxynucleosidetriphosphates (dNTPs) was studied in 9L cells. Pool sizes were determined by HPLC. In addition, the cytotoxic effects of didox and BCNU as single drugs and in equimolar combination were tested in 9L and in DAOY cells. Combination effects were determined according to the equation of Chou and Talalay. The expression of DNA repair-related genes was determined after exposure of 9L cells to BCNU, didox and a combination of the two compounds, using a cDNA array. *Results*: Incubation of 9L cells with 30 μ*M* didox for 24 h significantly decreased the intracellular concentrations of the DNA precursors dCTP (61% of control) and dGTP (17% of control), and significantly increased the concentration of dATP (155% of control). This dNTP imbalance compromised DNA synthesis and repair and might therefore have been, at least in part, responsible for the highly synergistic cytotoxic effects seen when BCNU was used simultaneously with didox in 9L and in DAOY cells. With almost all combinations tested, highly synergistic effects were seen, as indicated by combination indices of < 1 according to the equation of Chou and Talalay. In 9L cells, BCNU upregulated the expression of DNA repair-associated genes, whereas coincubation of the cells with didox reduced overexpression of some of these repair-related genes. *Conclusion*: A combination of BCNU and didox was proven to act in a synergistic manner in two cell lines, 9L rat gliosarcoma and DAOY human medulloblastoma cells. Further in vivo tests using these two compounds systemically and/or locally at the tumor site might be warranted.

Keywords Ribonucleotide reductase · Glioblastoma · Didox · BCNU · Synergistic combination chemotherapy

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Introduction

The enzyme ribonucleotide reductase (RR, EC 1.17.4.1) is responsible for the reduction of ribonucleosidediphosphates to their corresponding deoxyribonucleosides. The diphosphates are further phosphorylated to deoxynucleosidetriphosphates (dNTPs), which are the precursors of DNA synthesis. The activity of RR has been shown to be increased in malignant transformation and proliferation and is therefore considered an excellent target for cancer chemotherapy [5, 19, 20]. Inhibition of RR causes growth arrest of rapidly proliferating malignant cells and has little effect on slowly dividing, non-malignant cells.

One of the first RR inhibitors in clinical use was hydroxyurea, a drug which is mainly used for the treatment of leukemia patients. However, hydroxyurea is also used as one compound in various chemotherapeutic treatment protocols against human gliomas [11–13]. Other compounds such as fludarabine, or gemcitabine, are recently introduced and clinically effective inhibitors of the enzyme.

Newer inhibitors of RR are polyhydroxy-substituted benzohydroxamic acid derivatives, such as didox (3,4-dihydroxy-benzohydroxamic acid) or trimidox (3,4,5-trihydroxy-benzohydroxamide oxime) [22]. They are excellent free radical scavengers and have been shown to be active in vitro and in vivo against a number of human tumor cells, such as leukemia, colon tumor cells, breast cancer cells and human ovarian cancer cells [16, 19, 21]. Didox has even been tested clinically in phase I and II trials for advanced breast cancer with minimal toxic side effects [2, 17]. Both compounds, didox and trimidox, have been recently reported to be also effective against human glioma cells and to exhibit synergistic activity together with temozolomide, a recently introduced oral alkylating agent [7].

In the present study, we focused on the effects of didox on glioma cells. Gliomas are the most frequent primary tumors of the central nervous system and treatment of gliomas involves surgery, irradiation and chemotherapy [10]. More recently, local chemotherapy of these tumors has been attempted. Antitumor compounds can be delivered via implantable biodegradable polymers directly to the tumor site [14, 18]. For instance, local delivery of minocycline, a tetracycline derivative, which has been shown to inhibit tumor angiogenesis together with systemic BCNU (carmustine, 1,3-bis(2-chloroethyl)-1-nitrosourea) has demonstrated synergistic activity in the treatment of intracranial 9L gliomas in rats [8].

We therefore investigated the biochemical, cytotoxic and genetic effects of didox as a single compound and together with BCNU in 9L rat gliosarcoma cells and DAOY human medulloblastoma cells. BCNU was selected as it is an alkylating agent, like temozolomide, and might therefore yield synergistic effects when combined with didox. However, BCNU is the most frequently used antitumor compound for the treatment of malignant gliomas. Results obtained in this study should be the basis for the development of new combination treatment strategies. Further in vivo studies of didox as a single compound or in combination with other anticancer drugs administered systemically or locally after surgery might constitute the basis of an additional treatment option for malignant gliomas, which are still in most cases fatal.

Materials and methods

Chemicals

BCNU was purchased from Sigma (Munich, Germany). Didox was synthesized by Dr Bart van't Riet [22], and was generously provided by Molecules for Health (Richmond, Va.).

Cell culture

The 9L rat gliosarcoma cell line was derived from a tumor induced in the rat by *N*-nitrosomethylurea and can be utilized to generate an intracranial tumor model. It was obtained from Dr. Henry Brem and Betty Tyler at Johns Hopkins University (Baltimore, Md.). DAOY human medulloblastoma cells were purchased from ATCC (American Type Culture Collection, Manassas, Va.). 9L cells were grown in high glucose DMEM medium supplemented with 10% fetal calf serum, 1% glutamine and 0.5% penicillin/streptomycin. DAOY cells were grown in Improved MEM Zinc Option Medium supplemented with 10% fetal calf serum, 1% glutamine and 0.5% penicillin/streptomycin. The cultures were maintained in exponential growth phase in a humidified atmosphere containing 5% CO_2 at 37°C. All media and supplements were obtained from Gibco Life Technologies (Paisley, UK).

Analysis of intracellular dNTP pools by high performance liquid chromatography (HPLC)

The extraction of dNTPs from the cells was performed according to the method described by Garrett and Santi [9]. A total of 2×10^7 cells were seeded in 175-cm^2 flasks and after a 24-h preincubation period, the compounds were added. The cells were harvested after an incubation period of another 24 h and the dNTPs were extracted. All steps of the extraction process were carried out on ice. After adjusting for total cell count, the cells were centrifuged at 1800 rpm for 5 min and then resuspended in 100 µl phosphate-buffered saline. To this suspension was added 10 µl of trichloroacetic acid and the mixture was vortexed for 1 min. The proteins were separated by centrifugation at 15,000 rpm for 10 min in an Eppendorf microcentrifuge. The supernatant was removed and neutralized by adding a 1.1 volume of freon containing 0.5 *M* tri-*n*-octylamine. Samples were then periodated and dNTP concentrations were determined using HPLC (Merck, Germany) according to the method described by Garrett and Santi [9]. The experiment was repeated twice. The concentrations of dCTP, dTTP, dATP and dGTP in 2×10^7 exponentially growing 9L control cells were 7.8, 31.9, 5.0 and 4.9 µ*M*, respectively.

Analysis of overall genetic expression using cDNA arrays

A commercially available cDNA array, which contains 5000 known genes (Research Genetics, Huntsville, Ala.;

http://www.resgen.com) was used for testing genetic expression patterns. Total RNA was obtained from untreated control cells or cells after treatment with either drug alone and after incubation with an equimolar drug combination (50 μ*M* BCNU, 50 μ*M* didox) for 48 h. Total RNA from 2×10^6 9L cells was extracted using TRIZOL RNA extraction reagent (Gibco Life Technologies, Rockville, Md.) according to the manufacturer's protocol. The purity of the RNA was verified spectrophotometrically. A total of 1 μg RNA sample was used for reverse transcription. The RNA samples were primed and labeled using ^{33}P dCTP and reverse transcriptase, and the labeled probes were purified using Sephadex G50 columns (Boehringer-Mannheim). The labeling efficiency was verified by measuring the radioactivity of the sample prior to and after column purification. The cDNA membranes were prehybridized using 5 mg cot-1 DNA and 5 μl poly dA in MicroHyb solution (Research Genetics) for a minimum of 2 h. The purified sample was then added to the array membranes and hybridized for 18 h at 42°C. The membranes were washed twice with 2× SSC and 1% SDS for 20 min at 50°C followed by two washes with 0.5× SSC and 1% SDS for 15 min at room temperature. Analysis of filters following hybridization and washing steps was performed using a Storm Phosphorimager. The data were gathered using ImageQuant software, and were stored for analysis in digital image files. The images were quantified with MathLab/PScan software (Mathworks, Natick, Mass.) and transferred to Excel (Microsoft, Seattle, Wash.) worksheets. The statistical analysis of the data was performed using SPSS Base 8.0 with Advanced Statistics and Interactive Graphics packages (SPSS, Chicago, Ill.). The experiment was repeated twice and the results were calculated as percent change from untreated control cells. Genes that showed a significant increase or decrease compared to controls were selected after initial screening for further calculations.

Crystal violet cytotoxicity assay

The cells were plated in 24-well plates at a density of 0.1×10^6 cells/well and allowed to grow overnight at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, the medium was removed and replaced by fresh medium containing various concentrations of the drugs. The plates were incubated for 24 h with didox and BCNU alone and in combination at equimolar concentrations ranging from 10 to 500 μ*M* for 9L cells and 25 to 200 μ*M* for DAOY cells. After removing the medium from the wells, the plates were stained with 0.5% crystal violet solution for 15 min. The plates were then washed in distilled water by immersion and completely dried overnight. The absorbance was measured at 490 nm using an ELISA plate reader after adding 450 μl 10% SDS. Results were calculated as percent of control after correcting for blank absorption. Experiments were repeated three times.

Growth inhibition and cell viability assay

9L and DAOY cells were seeded at a concentration of 0.1×10^6 cells/ml in 25-cm^2 flasks and allowed to attach overnight. After incubation with 20, 50 and 100 μ*M* didox and BCNU alone and in combination at equimolar concentrations at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h, the cells were trypsinized and resuspended in complete medium. Then cell density and viability were determined by trypan blue staining. All samples were run in duplicate and all experiments were repeated at least twice. Change in cell density was calculated as percent of control cells.

Statistical calculations

Synergism between didox and BCNU was calculated according to the equation described by Chou and Talalay [3]. The results of experiments were analyzed and combination indices were calculated using CalcuSyn software (Biosoft, Cambridge, UK). The significance of differences were calculated using a *t*-test using Prism 3.0 software (GraphPad Software, San Diego, Calif.).

Results

Determination of deoxyribonucleoside triphosphates

After incubation of 9L rat gliosarcoma cells with 15 and 30 μ*M* didox for 24 h, dNTP pools were determined by HPLC, as described in the Methods section. The intracellular concentrations of dGTP, dCTP and dTTP decreased after incubation with didox, whereas the intracellular dATP pools significantly increased after 24 h of drug exposure. After incubation with 15 and 30 μ*M* didox, dGTP pools showed a significant decrease to 23% and 17% of control values. Intracellular dCTP concentrations decreased to 67% (n.s.) and 61% ($P=0.039$) and dTTP concentrations decreased to 85% (n.s.) and 67% (n.s.), respectively. Intracellular dATP concentrations showed an almost twofold, highly significant increase (186% and 155%) after incubation with 15 and 30 μ*M* didox, respectively. The results are presented in Fig. 1.

Analysis of overall genetic expression

9L rat gliosarcoma cells were incubated with 50 μ*M* BCNU or 50 μ*M* didox alone and with both compounds in combination for 48 h. BCNU caused a highly significant upregulation of a number of DNA repair-related genes, such as the nucleotide excision repair gene ERCC1, which showed the greatest increase of all studied genes after incubation with BCNU (274-fold). The damage-specific DNA binding protein 3 (DNA BP3) was also significantly (138-fold)

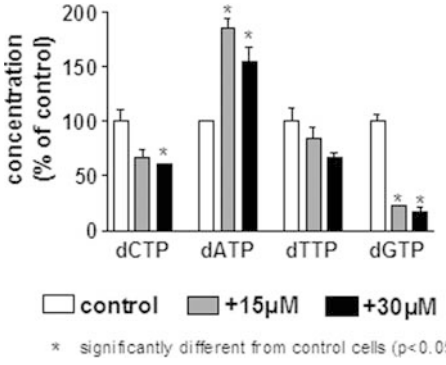

Fig. 1 Effect of didox (15 and 30 μM) on intracellular dNTP concentrations in 9L glioma cells. Cells were incubated for 24 h, then dNTPs were extracted and analyzed by HPLC. Data are means ± SEM of two determinations of one representative experiment

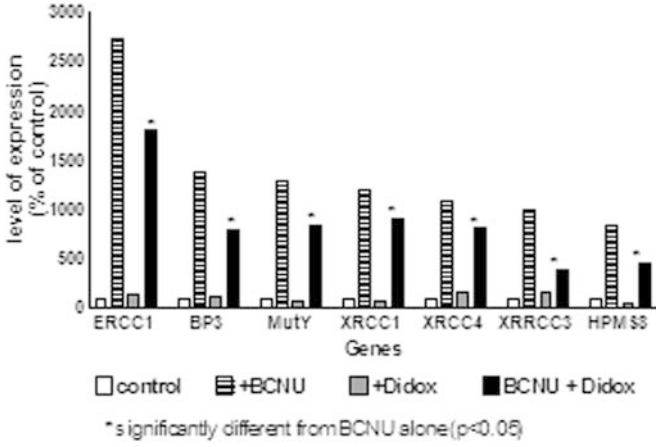

Fig. 2 Expression of DNA repair-related genes. Cells were treated with 50 μM BCNU and/or didox for 48 h, then total RNA was isolated. Data are the means of two determinations. Significant upregulation after BCNU incubation was observed in all examples, and these effects could be partially reversed by the addition of didox. *ERCC1* DNA repair protein ERCC1, *BP3* damage-specific DNA binding protein 3 (48 kDa), *MutY* MutY homolog, *XRCC1* DNA repair protein XRCC1, *XRCC4* DNA repair protein XRCC4, *XRRCC3* X-ray repair cross-complementing protein 3 (XRRCC3), *HPMS8* human DNA mismatch repair gene homologue HPMS8

upregulated after incubation with BCNU alone. The mammalian MutY homolog glycosylase gene showed a 128-fold overexpression after BCNU treatment and the genes coding for the DNA repair protein XRCC1 and the XRCC4, as well as the gene for the X-ray repair cross-complementing protein 3 (XRRCC3) were significantly (12-, 11- and 10-fold) upregulated following incubation with BCNU alone. Upregulation of the HPMS8 gene was also observed (9-fold), as shown in Fig. 2. Didox alone had hardly any effects on the expressions of the genes investigated. However, the upregulation of genes caused by BCNU alone could partially be reversed by coadministration of didox. These effects can be interpreted as a positive influence of didox incubation on repair-associated genes. The results are shown in Fig. 2.

Crystal violet cytotoxicity assay

Logarithmically growing 9L and DAOY cells were incubated with various concentrations of didox and BCNU alone and in combination at equimolar concentrations. In 9L cells, the IC_{50} values (concentrations resulting in a 50% inhibition of cell growth) after 24 h were 360 μM for didox, and 460 μM for BCNU. In DAOY cells, didox yielded an IC_{50} value of 160 μM and BCNU of 200 μM.

In 9L cells, combined incubation with equimolar concentrations of didox and BCNU yielded highly synergistic values for all combinations above 50 μM as shown in Table 1. Combination indices according to the equation of Chou and Talalay were all < 1, indicating synergistic combination effects. When the drugs were given sequentially at 3- or 6-h intervals, the effects were additive with BCNU as the first compound and less than additive with didox as the first compound (data not shown). In DAOY cells, didox and BCNU acted in a synergistic manner in all concentrations tested, with combination indices < 1. These results are shown in Table 2.

Growth inhibition assay

The cells were seeded in 5 ml medium at a concentration of 0.1×10^6 cells/ml. Cells were allowed to grow overnight, and were then incubated with 20, 50 and 100 μM didox and BCNU alone or with a combination of the two compounds. After 48 h of drug exposure, cell numbers and viability were determined. The results are presented in Figs. 3 and 4. In 9L cells, didox alone inhibited cell growth with an IC_{50} value of 70 μM. The IC_{50} value for BCNU was greater than 100 μM. The equimolar combination of the two drugs yielded an IC_{50} of 21 μM, indicating highly significant synergism with combination indices < 1. In the DAOY cell line, didox and BCNU yielded IC_{50} values of 55 and 50 μM, respectively. Synergism was also apparent at all three concentrations tested in this experiment. The equimolar combination of the two drugs yielded an IC_{50} value of 18 μM, and all combination indices were < 1.

Discussion

RR is the rate-limiting enzyme for DNA synthesis. In particular, in rapidly proliferating cells, the enzyme is responsible for de novo formation of dNTPs which are required for DNA synthesis [19]. Inhibition of RR therefore leads to growth inhibition and apoptosis of various tumor cells [16, 21] including human glioma cells. In the present study we investigated the combination effects of BCNU and didox. BCNU is an alkylating antitumor agent which is being used as a first choice drug for the chemotherapy of gliomas. Another group of

Table 1 Cytotoxic effect of BCNU and didox incubated as single drugs and in combination with 9L rat gliosarcoma tumor cells. The data are the means of two determinations, and standard deviations were within 5%. Cells were incubated with the drugs alone and in combination at equimolar concentrations for 24 h then absorbance was recorded. Predicted values were calculated as: didox×BCNU/100 (%)

Agent/combination	Concentration (μM)	Absorbance (% of control)	Predicted value (%)	Combination index
BCNU	10	99.6		
	20	98.6		
	50	85.0		
	100	73.5		
	200	59.3		
	500	48.5		
Didox	10	98.5		
	20	88.5		
	50	79.1		
	100	70.0		
	200	58.2		
	500	43.7		
BCNU + didox	10	90.8	98.1	2.00
	10			
	20	72.6	87.3	1.48
	20			
	50	22.1	67.2	0.19[a]
	50			
	100	22.4	51.5	0.18[a]
	100			
	200	8.2	34.5	0.02[a]
	200			
	500	0.1	21.2	0.02[a]
	500			

[a]Synergistic combination effect according to Chou and Talalay

Table 2 Cytotoxic effect of BCNU and didox applied as single drugs and in combination in DAOY human medulloblastoma cells. The data are the means of two determinations, and standard deviations were within 5%. Cells were incubated with the drugs alone and in combination at equimolar concentrations for 24 h then absorbance was recorded. Predicted values were calculated as: didox×BCNU/100 (%)

Agent/combination	Concentration (μM)	Absorbance (% of control)	Predicted value (%)	Combination index
BCNU	25	84.8		
	50	70.1		
	100	70.7		
	200	48.7		
Didox	25	81.5		
	50	64.1		
	100	62.8		
	200	40.7		
BCNU + didox	25	66.5	69.1	0.68[a]
	25			
	50	16.2	44.9	0.07[a]
	50			
	100	7.47	44.4	0.05[a]
	100			
	200	4.48	19.8	0.04[a]
	200			

[a]Synergistic combination effect according to Chou and Talalay

compounds that can be used for the treatment of gliomas are RR inhibitors. Hydroxyurea is being clinically used as part of a combination regimen and MDL101731, a novel ribonucleoside diphosphate reductase inhibitor, has been shown to inhibit the growth of glioblastoma and neuroblastoma in vitro and in vivo [15]. Didox, as mentioned before, is also an inhibitor of RR and has been shown to exhibit excellent growth inhibitory effects against human glioma cells. Figul et al. have recently reported that combinations of temozolomide, an oral alkylating agent, and the RR inhibitors didox or trimidox are cytotoxically highly synergistic [7]. One mechanism causing these synergistic effects was suggested to be the depletion of dNTP and as a consequence, inhibition of DNA repair after temozolomide-induced DNA damage. Indeed, we showed in the study reported here that didox significantly decreased dNTPs pool sizes in 9L glioma cells. In particular dCTP and dGTP pools decreased significantly after didox incubation, whereas dATP concentrations showed a significant increase. This imbalance of DNA precursors caused by didox inhibits DNA synthesis and/or DNA repair. One of the main mechanisms of action of BCNU is the induction of DNA damage [4]. Therefore, inhibition of DNA repair by depletion of dNTPs might be a possible mechanism of action for the observed highly synergistic effects of BCNU and didox. In addition, it is known that increased activity of enzymes linked to DNA repair can cause decreased sensitivity towards alkylating agents [1, 6]. We also showed that BCNU incubation increased the expression of a number of DNA repair-associated genes. The increased expression of some of these genes were

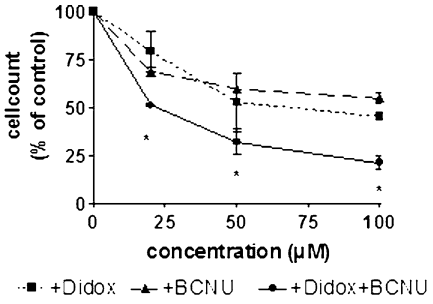

Fig. 3 Growth inhibition of 9L cells after incubation with didox and/or BCNU. Cells were incubated with the drugs for 48 h, and then the viable cells were counted. Data are means ± SEM of two determinations. Synergistic combination effects according to Chou and Talalay (combination index < 1) are marked with an *asterisk*

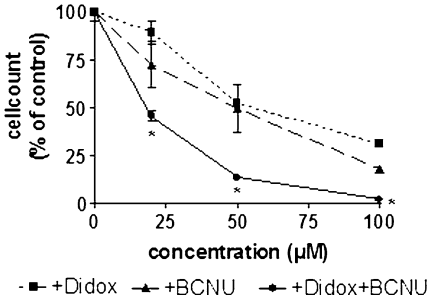

Fig. 4 Growth inhibition of DAOY cells after incubation with didox and/or BCNU. Cells were incubated with the drugs for 48 h, and then the viable cells were counted. Data are means ± SEM of two determinations. Synergistic combination effects according to Chou and Talalay (combination index < 1) are marked with an *asterisk*

reduced by coadministration of the ribonucleotide reductase inhibitor, didox.

Synergism was shown in two different brain tumor cell lines. Animal studies investigating local application of the drugs at the tumor site, as performed at Johns Hopkins University, Department of Neuropathology, might therefore be further warranted. These synergistic cytotoxic effects seen with the combination of BCNU and didox in gliosarcoma and medulloblastoma cell lines might be the basis for further studies with this drug combination.

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