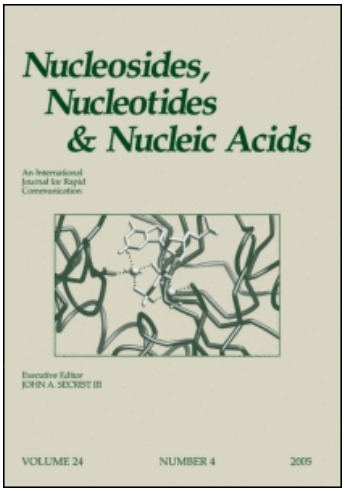


**Nucleosides, Nucleotides and Nucleic Acids**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 27 October 2004

**To cite this Article** Horvath, Zsuzsanna , Bauer, Wolfgang , Hoechtl, Thomas , Saiko, Philipp , Fritzer-Szekeres, Monika , Tihan, Tarik and Szekeres, Thomas(2004) 'Combination Chemotherapy of BCNU and Didox Acts Synergystically in 9L Glioma Cells', Nucleosides, Nucleotides and Nucleic Acids, 23: 8, 1531 — 1535
**To link to this Article: DOI:** 10.1081/NCN-200027746
**URL:** http://dx.doi.org/10.1081/NCN-200027746

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 8 & 9, pp. 1531–1535, 2004

# Combination Chemotherapy of BCNU and Didox Acts Synergystically in 9L Glioma Cells

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## ABSTRACT

Compounds inhibiting DNA repair and synthesis are expected to act synergistically with BCNU, a standard agent in the therapy of glioblastoma multiforme, and improve survival of patients with malignant gliomas. Ribonucleotide reductase (EC1.17.4.1; RR) catalyzes the rate-limiting step in DNA synthesis and plays a critical role in maintaining crucial substrates for DNA repair. We have studied the effects of Didox, an inhibitor of RR on 9L glioma cells in combination with BCNU. We analyzed intracellular dNTP pools and found that Didox significantly depleted the intracellular dNTP concentrations. Experiments using cytotoxicity, growth inhibition and clonogenic assays showed significant synergism of Didox and BCNU. Combination regimens using synchronous administration demonstrated highest cytotoxicity. We have also identified altered gene expression in a number of DNA repair related enzymes after BCNU treatment using large-scale cDNA arrays. The coadministration with Didox could reverse the expression of some of the overexpressed repair gene suggesting possible pathways to circumvent the developing resistance in 9L glioma

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DOI: 10.1081/NCN-200027746
1525-7770 (Print); 1532-2335 (Online)

cells against BCNU. These results introduce the combination of Didox and BCNU as a viable alternative for the treatment of malignant gliomas.

*Key Words:* Glioblastoma multiforme; Ribonucleotide reductase; Combination chemotherapy; BCNU; Didox.

## INTRODUCTION

A significant number of malignant gliomas develops resistance to 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) therapy, a first-choice drug in adjuvant chemotherapy. Increases in DNA repair and synthesis are implicated as the main causes of BCNU resistance.[1] A possible alternative to improve the efficacy of BCNU treatment is the design of combination chemotherapy regimens that are synergistic. Compounds inhibiting DNA repair and synthesis are expected to act synergistically with BCNU. Ribonucleotide reductase (EC1.17.4.1; RR) catalyzes the rate-limiting step in DNA synthesis and plays a critical role in maintaining crucial substrates for DNA repair. The increased rate of RR expression in cancer cells makes this enzyme an excellent target in cancer chemotherapy. The benzohydroxamic acid derivative Didox (3,4-dihydroxybenzohydroxamic acid) is a potent inhibitor of RR, and has been proven to induce apoptosis in vitro and in vivo.

Therefore we have studied the effects of Didox on 9L glioma cell line in combination with BCNU.



## MATERIALS AND METHODS

### Chemicals, Supplies, and Cell Culture

BCNU was purchased from SIGMA (Munich, Germany). Didox (3,4-dihydroxybenzohydroxamic acid) was synthesized by Dr. Bart van't Riet[2] and was provided by Molecules for Health (Richmond, VA).

The 9L rat gliosarcoma cell line was obtained from Dr. Henry Brem and Betty Tyler at Johns Hopkins University (Baltimore, MD). The cells were grown in high glucose DMEM 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, Ltd. (Paisley, Scottland).

### Analysis of Intracellular dNTP Pools by High Performance Liquid Chromatography

The extraction of dNTPs from the cells was performed according to the method described by Garrett et al.[3]

### Crystal Violet Cytotoxicity Assay

9L cells ($10^5$/well) were incubated in 24-well plates with Didox and BCNU alone and in combination with concentrations ranging from 10 to 500 μM for 24 hours. After

removal of medium, plates were stained with 0.5% crystal violet solution for 15 min. The plates were then washed and 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.

### Analysis of Overall Genetic Expression Using cDNA Arrays

This group of analyses utilized a commercially available cDNA array that contains 5000 known genes (Research Genetics Inc. Huntsville, AL; http://www.resgen.com.). Total RNA was isolated from 9L cells with TRIZOL RNA extraction reagent (Gibco Life Technologies, Ltd. Rockville, MD) and was analyzed according to the manufacturer's protocol.

## RESULTS

### Analysis of Intracellular dNTP Pools

Didox significantly depleted the intracellular dNTP concentrations (Fig. 1). After incubation with 15 and 30 µM Didox, the intracellular concentrations of dGTP fell to 34 and 16% of control values. dCTP and dTTP pools also showed significant decrease (66 and 61% in the case of dCTP and 84 and 67% in the case of dTTP, respectively). The intracellular dATP pools have increased to 186 and 154% after treatment with 15 and 30 µM Didox, respectively.

### Crystal Violet Cytotoxicity Assay

In crystal violet cytotoxicity assays, BCNU showed significant synergism with Didox. After a combined treatment using equimolar concentrations of the two drugs between 10 and 500 µM, a combination index <1 was reached in concentrations between 50 and 500 µM (Fig. 2). Cell kill was greatest when the two substances were applied simultaneously.

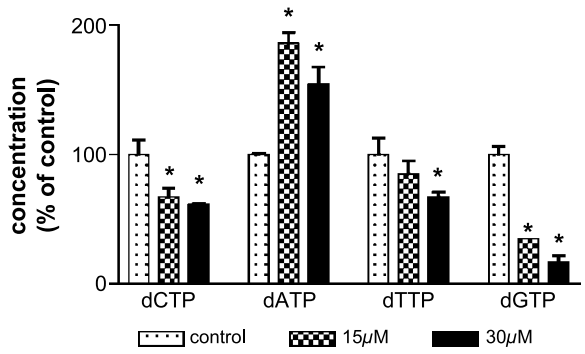


***Figure 1.*** Effect of Didox treatment on intracellular dNTP concetrations in 9L glioma cells. All values are sigificantly different from control, experiment was repeated twice. *Significantly different from control.

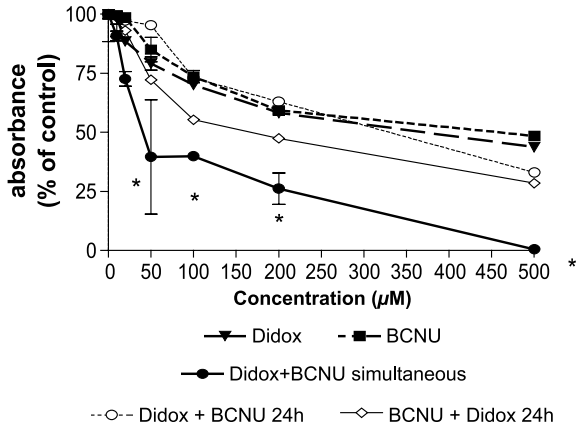


***Figure 2.*** Cytotoxic effects of BCNU and Didox alone and in combination in 9L glioma cells as measured by the crystal violet assay. *Synergistic combination index.



## Analysis of Overall Genetic Expression

We have also identified altered gene expression in a number of DNA repair related enzymes using large-scale cDNA arrays, suggesting possible pathways to circumvent the developing resistance in 9L glioma cells against BCNU. A number of genes was associated with excision repair mechanisms (ERCC1) but other pathways of DNA repair were also involved (Human DNA mismatch repair gene homologue HPMS8). BCNU caused a significant upregulation of these genes, but the coadministration of Didox could partially reverse this effect, counteracting developing resistance to BCNU. We have tested numerous genes associated with DNA repair, some were upregulated, such as DNA polymerase gamma (179% of control), DNA repair protein ERCC1 (132%), damage specific DNA binding protein 3 (127%), DNA repair protein XRCC4 (170%), X-ray repair cross-complementig protein 3 (XRRCC3, 165%), in other cases we could observe a downregulation by Didox treatment only, such as human DNA mismatch repair gene homologue HPMS8 (57%), DNA polymerase epsilon subunit (61%) and ATP-dependent DNA Ligase (34%). The effects of other RR-inhibitors was not tested in this set of experiments.

## DISCUSSION

In our experiments, we showed that Didox effectively decreases intracellular dNTP concentrations, and can interfere with DNA repair mechanisms. In cytotoxicity studies, Didox acted synergistically with BCNU and effectively inhibited the growth of 9L glioma cells in vitro. Didox was also able to downregulate the expression of a number of genes linked to DNA-repair mechanisms.

The serum level which can be reached by intravenous administration is 300 μM for Didox. In the case of BCNU, there exist implantable biodegradable polymers, which are applied in the tumor cavity. The concentration of BCNU delivered by

this system lies in the micromolar range. These concentrations are in the range of our experiments.

Therefore, our results introduce the combination of Didox and BCNU as a viable alternative for the treatment of malignant gliomas.

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