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In vitro analysis of the cellular resistance to chemotherapeutic BCNU

A. Caballero Navarro[1], B. Conde Guerri[2], E. Sinues Porta[2], E. Boada Apilluelo[1] and A. Alcalá Arellano[2]

[1]Department of Physiatry and Nursery and [2]Department of Morphological Sciences, Faculty of Medicine, University of Zaragoza, Zaragoza, Spain

Summary. Our asays in vitro show that BCNU inhibits cell proliferation in the C_6 cell line experimental glioma and is dose-dependent, starting from 0.5 µg/ml of the drug with just an hour of exposure.

For every tested concentration of BCNU it is shown that, from the fifth day after exposure, cellular resistance appeared. This resistance is justified by the capacity of cell DNA reparation. A study of the clonogenic capacity of the C_6 cells exposed to BCNU also shows the appearance of cellular resistance for doses of 0.5 µg/ml and 1 µg/ml.

Furthermore, the exposure of C_6 cell cultures to BCNU at these levels produces a cellular evolution towards more differentiated morphological patterns.

Key words: Chemotherapy, BCNU, Cellular resistance, Glioma

Introduction

A chemotherapeutic agent can have a variety of mechanisms and due to them a cell can be resistant. It is difficult to explain drug resistance by a single mechanism alone, since drug resistance differs depending of the type of tumour and the drug (Yoshida et al., 1987).

Cellular resistance has been described associated with increased capacity for DNA repair. This kind of resistance has been shown mainly for the alkylating agents and Busulfan (Bodell et al., 1985, 1988; Aida and Bodell, 1987).

The carcinogenic, mutagenic and cytotoxic effects induced by nitrosoureas (BCNU, CCNU) with obvious injury of cellular DNA, can be modified by the capacity for DNA repair, apart from the repair done by the enzyme 0_6 Methyldeoxiguanosine (Young, 1990).

This capacity for repairing the DNA interstrand cross-link formation can be demonstrated with drugs such a Busulfan and Cisplatinum, beside the Nitroseureas (Ericson et al., 1980).

Several studies have implicated several enzymes in the appearance of cellular resistance to drugs such a BCNU and Nitrogen Mustard (Evans et al., 1987).

The fast increase of our understanding of the molecular bases of drug resistance in human tumours will probably generate additional clinical strategies to prevent or reserve this phenomenon.

Materials and methods

Biological material. The C_6 cell line was obtained from Flow laboratories and cultured with Ham's F_{12} supplemented with 10% Bovine Fetal serum and 1.000 i.u. of sodium penicillin. The monolayer cultures were made in Falcon 25cm2 (Greiner) flasks with 6 ml of medium.Single cell suspensions were obtained by incubation of confluent cultures with Trypsin + EDTA (2 ml) in each flask for one minute.

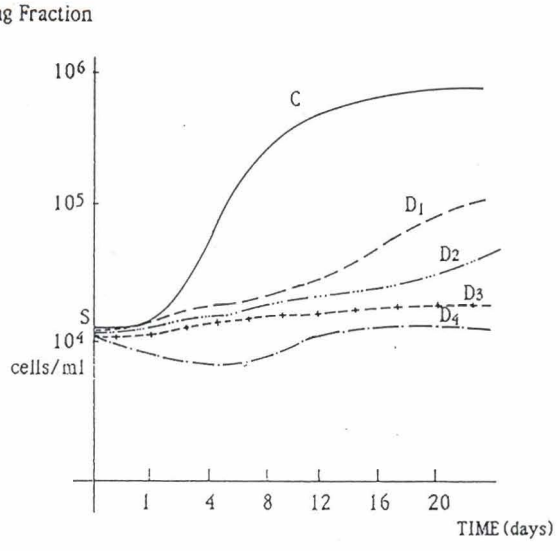
BCNU (Nitrosourea). Obtained from the Bristol-Myers laboratories (Syracuse, N.Y.), in vials of 100 mg Carmustine, with a vial of 10% ethanol in 3 ml of solvent. The final solution of BCNU was equivalent to a concentration of 3.3 mg/ml.

Methodology.

The C_6 cells were plated in five series in Falcon flasks, in a concentration of 1 x 10^4 cells/ml. When the cultures were in the stage of exponential growth, they were treated with various concentrations of BCNU (0.5, 1.5, 2 and 4 µg/ml) for 1 hour at 37º C, after which the medium in all flasks was removed.

Later on, the chemotherapeutic agent was removed and the cultures were again incubated (previously

Offprint requests to: Dra. Dª Ana Caballero Navarro, C/ San Juan de la Cruz nº 9, 4º D, 50006 Zaragoza, Spain



S: Density of cellular seeding (3 • 10^4 cells/ ml)
C: Control
D_1: Dose 1 BCNU = 0.5 µg/ml
D_2: Dose 2 BCNU = 1.5 µg/ml
D_3: Dose 3 BCNU = 2 µg/ml
D_4: Dose 4 BCNU = 4 µg/ml

Fig. 1. Dose/Response curves of cell line C6 exposed to different doses of BCNU (two consecutive exposures).

treated with BCNU in a 5% CO_2 - 95% air atmosphere for 4 or 5 days). After this time, a second exposure to BCNU was made for 1 hour and at the same levels as described.

Assays for cellular proliferation. Curves of cellular viability (Gerosa et al., 1986): Obtained by sequenced cell counting, with Trypan Blue stain and a hemocytometer, every four days for 20 days.

Analysis of the replicative capacity of cellular DNA. Technique of 5-BrdU (Dolbeare et al., 1983; Hoshino et al., 1986): By immunocytochemical detection of 5-bromodeoxy-uridine that incorporated specifically in the S-phase of the cell cycle.

Microphotographies were taken of those nuclei which showed incorporation of 5-BrdU with intranuclear granulations, with a Phaco and a Kodak-Ektachrome objective (x 40) (23 DIN, 160 ASA film) and/or (Tri-x Pan, 27 DIN, 400 ASA) Kodak.

The dose of 5-BrdU incorporated to the cultures was 1 µl/ml (Sigma-Chemicals, St. Louis, MO). It was maintained in the cultures for 30 minutes.

After washing the cultures, they were incubated with FITC-anti BrdU (Becton-Dickinson) monoclonal antibody, diluted 1:30 for one hour at 37º C in a humid chamber.

Clonogenic assays, C.F.A. (Colony Forming Assay). Colonies were cloned starting from cultures, after one and two treatments with BCNU and 13 days after removal of the drug (Weisenthal et al., 1983).

Studies of cellular differentiation. Morphological changes in C_6 cells subjected to two treatments of BCNU, were determined examining the cultures periodically with a phase-contrast microscope (Leitz-Diavert).

Results

Proliferating capacity.

Growth curves (BCNU dose - response): The cultures exposed to BCNU levels of 0.5, 1.5, 2 and 4 µg/ml respectively and for two consecutive treatments showed a slight increase in the cellular population (starting with the 8th day of treatment) (Fig. 1). From the 4th to the 8th day, the culture population was stable (Tables 1 and 2).

Nevertheless, at 2 and 4 µg/ml of BCNU, the slight increase in cellular growth disappeared from the 12th day and showed growth inhibition of 92% in the last tested days.

With doses of 0.5 and 1.5 µg/ml there were clear signs of cellular recovery, possibly induced by the appearance of cellular resistance to BCNU from the 8th day after exposure to this chemotherapeutic agent. This phenomenon was not evident at 4 µg/ml.

Analysis of the replicative capacity of DNA: In cultures with the maximal level of BCNU (4 µg/ml) no cellular population with replicative capacity of DNA was found because the results obtained were:

- captation percentage of 5-BrdU = 0%
- intensity of labelling: nil. There was no evidence of intranuclear granulations. L.I. = 0.

There were analogous responses at all levels tested. In the control culture L.I. = 100 ± 1 (40 - 50 granulations per nucleus).

Clonogenic capacity. C.F.A. In spite of the time elapsed between both experiments (20 days) and after two successive treatments with BCNU at the same levels, the C6 cell cultures still showed clonogenic capacity with an Index of Plating Efficiency of:

- Control: I = 1
- Dose 1: I = 0.71
- Dose 2: I = 0.26
- Dose 3: I = 0.097
- Dose 4: I = 0.032

With doses 3 and 4, however, this capacity suffered a significant decrease when evaluated after the second exposure to Nitrosourea (Table 3).

Cellular differentiation. In the cultures exposed to the maximum concentration of BCNU, morphological changes were found 24 hours after treatment. There was

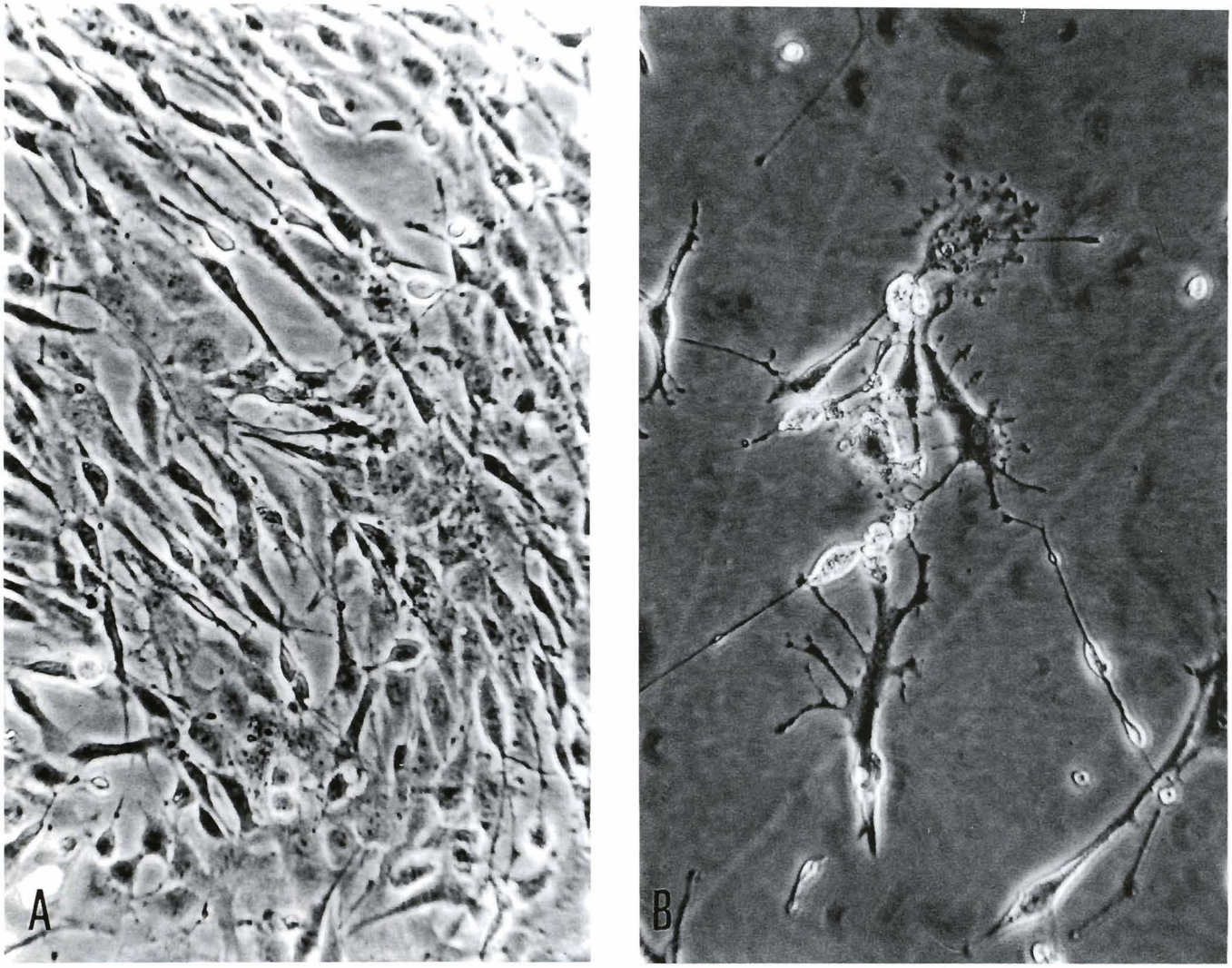


Fig. 2. Morphological changes in line C6 exposed to BCNU. **A.** Cell line C_6. Control. **B.** Cell line C_6 with BCNU (Dose 2 = 1.5 mg/ml.) Phase ontrast microscope (x 250)

an increase in the nucleus/cytoplasm ratio and some figures of isolated mitosis. Devitailized cells were also seen in small numbers and with imprecise cellular limits at all levels studied.

These morphological changes were reversible two and three days after the first treatment with BCNU.

After the second treatment the morphological alterations were much more intensitive and were found to be irreversible (Fig. 2).

Discussion

In all the asays performed to evaluate the proliferative capacity of the C_6 line, under the effects of BCNU, we observed that in a short space of time and from the first hour, there was an inhibition in cellular growth in all the cultures exposed to the various concentrations of the drug.

However, from the 5th day a slight, but progressively increasing growth (at all doses) was seen in the surviving cell fraction.

This leads us to think that two phenomena happened in these cultures:

- an evident dose-dependent cytotoxic effect from the first hour following drug exposure
- the appearance of cellular resistance to Nitrosourea from the 5th day after the treatment, including the maxima cell concentration assayed. We deduce that there is some mechanism for repair of the damage caused by BCNU, that becomes evident from this day and that the injuries produced by this drug can be reversed.

Weinkam and Deen (Weinkam and Deen, 1982) proved a relationship in the dose-response of cytotoxicity of Chlorethylnitrosureas in cultures of the 9L cell line murine gliosarcoma experimentally induced

Table 1. Proliferative capacity of the cell line C6 exposed to different doses of BCNU. Assays of cellular resistance to BCNU (Two consecutive exposures).

	Number of cells x 10^4				
Day 1	4	8	12	16	20
Control. 22.75 ± 7.13	67.8 ± 1.6	103.5 ± 7.2	104.3 ± 5.1	105.6 ± 2.1	100 ± 1.1
Dose 1. 29.3 ± 3.3	30 ± 0***	32.3 ± 3.4*	36.2 ± 3.5**	0.7 ± 3.7**	47.3 ± 3.8**
Dose 2. 11.2 ± 2.4	12.1 ± 2.4***	15.6 ± 2.7***	18 ± 0***	20.5 ± 3.02***	19.2 ± 2.2***
Dose 3. 10 ± 0	10.6 ± 2.3***	12 ± 0.3***	12 ± 0***	12 ± 2.5***	10. ± 0.1***
Dose 4. 7.25 ± 1.9	8.6 ± 2.1***	10 ± 2.3***	10 ± 0.1***	8 ± 0***	8 ± 2.07***

The numbers shown in the table are the median ± the standard deviations of the registered values.

NS : $p > 0.05$ (Non significative)
* : $p = 0.05$
** : $p > 0.01$
*** : $p = 0.001$

Table 2. Fraction of growth in the cell line C6 exposed to different doses of BCNU. Assays of cellular resistance to BCNU (Two consecutive exposures).

	(Expressed as percentages)				
Day 1	4	8	12	16	20
Control. 34.06	37.16	19.25	8.07	10.21	9.57
Dose 1. –	29.02	31.40	35.35	39.86	46.46
Dose 2. –	11.17	14.88	17.13	19.62	18.18
Dose 3. –	9.65	11.11	11	12.07	8.70
Dose 4. –	7.75	9.14	9	6.75	7

with N-methylnitrosurea. The authors observed that antitumoral activity of these agents is related to the alkylating and carbamylative capacity of the intermediate products that are formed by degradation of the original «mother» compound under certain physiological conditions (Montgomery, 1976). The alkylation of certain macromolecules in the cell, such as DNA, is responsible for the antitumoral effect of these agents. The initial reaction of chlorethylation may produce DNA interstrand cross-link formation, that would explain the mechanism of action of this drug (Ericson et al., 1980).

It has been proved that enzymes such as glutathione-transferase may play an important role in the cellular resistance to BCNU in the 9L cell line (Evans et al., 1987) derived from rat gliomas. However, the authors demonstrated that there were equal levels of the enzyme in sensitive (9L) and resistant (9L-2) lines. There was, however, on the resistant lines (9L-2) an increase in the repairing by the enzyme O_6 chloroethylguanina, which is found in a higher quantity than in sensitive lines (Bodell et al., 1985).

Our results obtained with the Coloning Forming Assay with C6 cells exposed to the BCNU support those published by Yung on two cellular lines derived from human glioblastomas (RP and WF) exposed to higher concentrations of BCNU (20, 30, 40, 60, 80, 120 and 240 µg/ml of medium (Yung et al., 1982).

We concur with other authors that cellular resistance to BCNU developed mainly during the exposure to low

Table 3. Clonogenic capacity of the cell line C6 exposed to different doses of BCNU.

First treatment. Assays of cellular resistance to BCNU.

	Number of colonies	T-test
	X ± S.D.	
CONTROL	108 ± 4.68	
DOSE 1	75.2 ± 4.32	0.035*
DOSE 2	70.6 ± 4.24	0.05*
DOSE 3	69.3 ± 4.23	0.029**
DOSE 4	40.25 ± 3.69	0.01**

Second treatment. Assays of cellular resistance to BCNU.

	Number of colonies	T-test
	X ± S.D.	
CONTROL	108 ± 4.68	
DOSE 1	80.3 ± 4.38	0.042**
DOSE 2	28 ± 3.33	0.01**
DOSE 3	10.5 ± 2.35	0.0099***
DOSE 4	3.5 ± 1.25	0.0085***

The numbers shown in the table are the median ± the satndard deviation of the registered values.

* : $p = 0.05$
** : $p < 0.01$
*** : $p = 0.0001$

P : probability statistically significant with respect to the control group according to Student's t-test.

concentrations of the drug. Besides, the heterogeneous chemosensitivities of different cell populations of glioma represent a very important factor to be taken into account when designing programs of chemotherapy in patients with malignant gliomas (Rosenblum et al., 1983).

BCNU also rapidly induces a marked cell differentiation which is manifested in glial morphological patterns as well as in the maturation scale of the cytoskeleton (Benda et al., 1971: Rubinstein et al., 1984).

The resistance of brain tumours to BCNU could be modified by agents which inhibit the repair mechanism of damaged cellular DNA and reinforce the lethal effects of Nitrosoureas. The administration of such agents may allow more effective treatment of brain and other solid tumours.

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