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Short Communication

THE ANTITUMOR DRUG, 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA, INACTIVATES HUMAN NICOTINAMIDE MONONUCLEOTIDE ADENYLYLTRANSFERASE

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Abstract—Nicotinamide mononucleotide (NMN) adenylyltransferase (EC 2.7.7.1) from human placenta is rapidly inactivated by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). A similar inactivation is observed with other *C*- and *N*-nitroso compounds. The inactivation by BCNU is dependent on incubation time, temperature and BCNU concentration. Protective reagents for -SH groups, dithiothreitol and β-mercaptoethanol, and the substrate NMN are very effective in protecting NMN adenylyltransferase from BCNU inactivation and in preserving its catalytic properties, while ATP is less efficient. Incubation of BCNU-inactivated and dialysed NMN adenylyltransferase with dithiothreitol results in a partial recovery of the enzymatic activity.

Key words: NMN adenylyltransferase; BCNU; NAD biosynthesis; enzyme inhibition

During the last three decades a whole range of clinically effective antitumor drugs have been developed; many of these act directly on the tumor cell, whereas others must be activated by metabolic processes, occurring both in the tumor cell and in organs such as liver [1].

The biochemical actions of these cytotoxic agents have been intensively investigated but comparatively little is known about the precise way in which they kill normal and malignant cells. Rice *et al.* [2] recently reported that the selective killing of leukemic and other malignant human cells due to *C*-nitroso drugs must be ascribed to the inactivation of the enzyme poly(ADP-ribose) polymerase leading to the derepression of DNA degradation by the nuclear calcium/magnesium-dependent endonuclease. Since this endonuclease is maintained in a latent form by poly(ADP-ribosyl)ation, the abolition of such covalent modification due to the inactivation of poly(ADP-ribose) polymerase by the action of C-nitroso compounds derepresses the DNA degrading activity. The authors presume that the tumor cell specificity of the cytocidal action of C-nitroso drugs may depend at least on two causes: (i) the probably malignant cell type-specific molecular vicinity of poly(ADP-ribose) polymerase to the calcium/magnesium activated endonuclease within the chromatin, facilitating an inhibitory interaction; and (ii) the special structural features of DNA itself in malignant cells. Poly(ADP-ribose) polymerase is thus identified as a critical regulatory enzyme component of a DNA-binding multiprotein system that plays a central role in defining DNA structures in the intact cell.

In order to study the possible involvement of NAD biosynthetic pathway in the cellular response to such compounds we have examined the *in vitro* effect of several nitroso compounds on the activity of human NMNAT‡ which converts NMN and ATP to NAD and PP_i [3]. The present work deals with the results obtained with BCNU, an *N*-nitroso compound and a chemotherapeutic agent with proven clinical activity against multiple myeloma and other human tumors [4–6].

Materials and Methods

Materials. BCNU was purchased from Sintesa S.A. (Belgium); NMN, ATP, NAD, and nitroso-compounds were obtained from the Sigma Chemical Company (St. Louis, MO, U.S.A.). All other reagents and materials were of the highest quality commercially available.

Purification of NMN adenylyltransferase. NMNAT from human placenta with a specific activity of 9.5 μmol/min per mg was used. The homogeneous enzyme was obtained by the classical chromatographic procedure, as previously described [7]. The final preparation was stored at 4° until use.

Enzyme activity assay. Enzyme activity was tested by a continuous spectrophotometric coupled enzyme assay [8]. The standard reaction mixture consisted of 60 mM HEPES buffer, pH 7.6, 1.18 mM NMN, 1.47 mM ATP, 20.7 mM $MgCl_2$, 35 mM semicarbazide hydrochloride, 0.45% (v/v) ethanol, 7.8 U of yeast alcohol dehydrogenase, 0.59 mg/mL bovine serum albumin, and an appropriate amount of sample to be assayed, in a final volume of 0.85 mL. The reaction was started by the addition of NMN and continuously followed at 340 nm in a Beckman DU-70 spectrophotometer. The temperature was maintained at 37°. In an alternative assay procedure, enzyme activity was

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‡ Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DTT, dithiothreitol; β-ME, β-mercaptoethanol; NMNAT, NMN adenylyltransferase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, NMN, nicotinamide mononucleotide; PP_i, inorganic pyrophosphate.

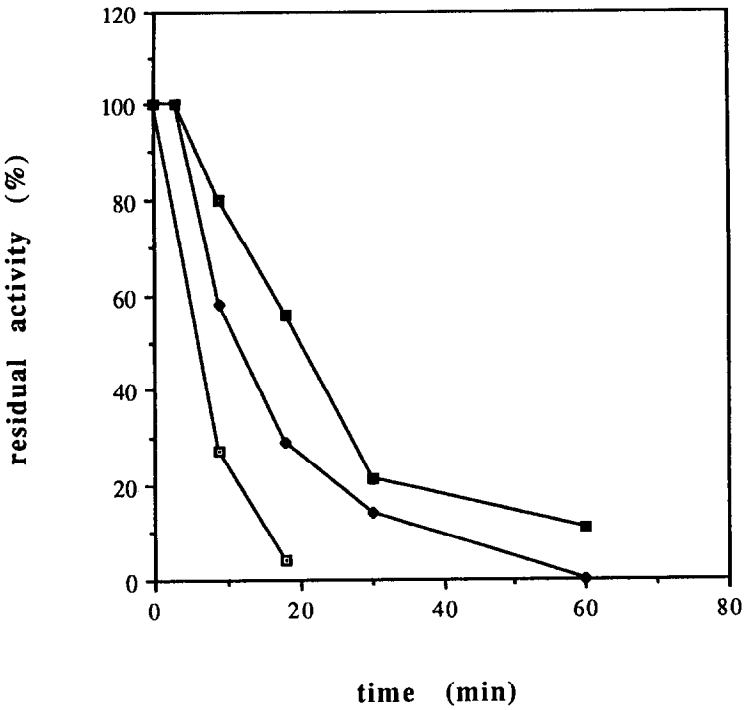

Fig. 1. Inactivation of NMN adenylyltransferase from human placenta by BCNU. The enzyme (9 mU) was treated with 0.5 mM (–■–), 1.3 mM (–◆–) and 2.8 mM (–□–) BCNU, and the residual activity was determined at the indicated times, as described in Materials and Methods. Conditions were: 70 mM Tris buffer, 28 mM $MgCl_2$, pH 7.5, 37°. The enzyme was incubated with 50 μL of appropriately water diluted BCNU. At various time intervals, samples were withdrawn from the incubation mixture and the residual activity expressed as per cent fraction of reference sample activity. Each data point represents the mean of three independent experiments, made in duplicate.

measured by HPLC quantitation of the reaction products and substrates [9].

One enzyme unit is defined as the amount of enzyme that catalyses the synthesis of 1 μmol of NAD per minute at 37°.

Results

Inactivation of NMNAT with BCNU. BCNU was tested for its ability to affect human homogeneous NMNAT activity. At room temperature the inactivation of the enzyme proceeded at a very low rate. Therefore, to monitor the time-dependent profile of enzymatic activity, the reaction was carried out at 37°.

The results obtained from the enzyme inactivation experiments at various concentrations of BCNU are presented as a plot of residual activity (as per cent fraction of reference sample activity) versus time (Fig. 1). These plots, obtained by analysing NMNAT activity in the forward direction (NAD synthesis), indicate that the loss of activity, under the conditions used, is dependent both on the incubation time and the BCNU concentration. This time-dependent fall in enzymatic activity is very sharp during the first 30 min, while, after this period, it occurs at a lower rate. Incubation of NMNAT with BCNU for 60 min, at a concentration lower than 0.25 mM (not shown), did not result in any significant loss of enzyme activity.

Protection of NMNAT against inactivation. If the substrates ATP and NMN were included in the inactivation mixture they were able to protect the enzyme activity against BCNU (Table 1). NMN exerted complete protection at a very high nucleotide concentration, which greatly

Table 1. Protective effects of ATP and NMN against inactivation of NMN adenylyltransferase from human placenta by BCNU*

Treatment	Residual activity (%)
None	100
BCNU (2.8 mM)	4
BCNU (2.8 mM) + ATP (1.7 mM)	61
BCNU (2.8 mM) + ATP (3.0 mM)	71
BCNU (2.8 mM) + NMN (0.5 mM)	70
BCNU (2.8 mM) + NMN (1.4 mM)	89
BCNU (2.8 mM) + NMN (2.9 mM)	100

* The enzyme (9 mU) was treated with BCNU (2.8 mM) for 20 min at 37° in the presence of ATP (1.7 mM, 3.0 mM), and NMN (0.5, 1.4 and 2.9 mM) and then the residual activity was assayed. The samples were supplemented by different amounts of ATP and NMN, taking into account the substrate concentrations already present in the inactivation mixtures, so that the final concentrations were the same, as described in Materials and Methods.

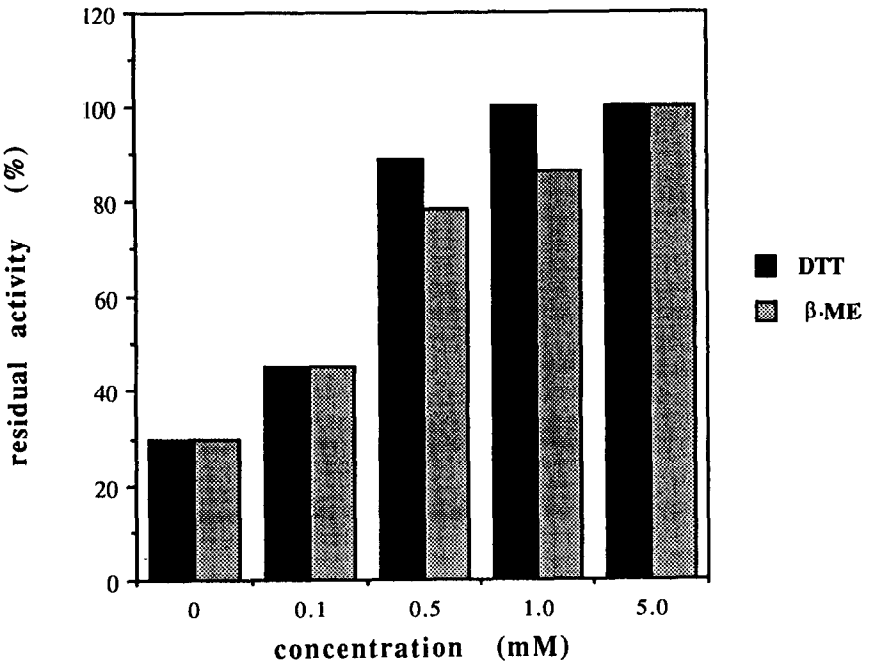

Fig. 2. Protective effects of DTT and β-ME against inactivation of NMN adenylyltransferase from human placenta by BCNU. The enzyme (9 mU) was treated with BCNU (1.3 mM) for 20 min at 37° in the presence of different concentrations of DTT and β-ME and the residual activity in the incubation mixture was assayed as described in Materials and Methods. The residual activity is referred to reference sample activity taken as 100%. Results are given as means from three independent experiments.

exceeded the K_m value for NMN (38 μM). At lower concentrations of NMN, the enzyme was protected to a lesser extent. The other substrate ATP is less efficient than NMN; indeed the presence of high ATP concentration did not completely overcome the inactivation exerted by BCNU. Figure 2 shows the effect of protective reagents for SH groups, DTT and β-mercaptoethanol, on NMNAT activity with respect to BCNU inactivation. At a concentration of 1 and 5 mM, respectively, DTT and β-mercaptoethanol were able to completely preserve NMNAT activity from BCNU action. The results obtained suggest that the preservation of the catalytic capacity of NMNAT depends on the integrity of the -SH group's reduced form.

Reversibility of enzyme inactivation. Removal of BCNU through exhaustive dialysis did not result in any recovery of NMNAT activity, suggesting that the loss of enzymatic activity following BCNU treatment is caused by a permanent modification of NMNAT. Incubation of BCNU-inactivated and dialysed NMNAT, with DTT (100 mM), as described in Table 2, resulted in a partial recovery of enzymatic activity. This recovery was temperature and dithiothreitol concentration dependent; indeed both at 25° and at low concentration dithiothreitol did not restore NMNAT activity in the presence of NMN (4.3 mM). The restoring process, DTT-mediated, was instantaneous. This partial recovery of enzymatic activity with DTT indicates that the modification of NMNAT thiol groups could be only one of the mechanisms underlying the observed effect of BCNU on enzyme activity.

Inactivation of NMNAT with other C- and N-nitroso compounds. Table 3 shows the effect of other *C*- and *N*-nitroso compounds on the activity of NMNAT. NMNAT activity was evaluated by analysing the reaction in both the forward and the reverse direction. Nitrosobenzene significantly depressed enzymatic activity, while MNNG appeared less effective. The results indicate that the loss of activity is dependent on incubation time, temperature and nitroso compound concentration (not shown), as obtained for BCNU.

Table 2. DTT effect on BCNU-inactivated NMN adenylyltransferase*

Addition	Residual activity (%) 25°	37°
None	40	39
DTT (5.4 mM) + NMN (4.3 mM)	41	40
DTT (100 mM)	40	60

* The enzyme (9 mU), in Tris buffer, pH 7.5, containing 28 mM $MgCl_2$, was first treated with BCNU at 37° until 60% of inactivation and then dialysed for 24 hr at 4° against the same mixture medium, BCNU free (0.5 l, four times). After dialysis DTT (100 mM) or DTT (5.4 mM) plus NMN (4.3 mM) were added; the mixtures were then incubated at 25° and 37°, respectively, and the enzymatic activity was evaluated spectrophotometrically, as described in Materials and Methods.

Discussion

The antitumor drug BCNU is one of the most extensively employed cancer chemotherapeutic agents in use today for a variety of tumor types, including multiple myeloma [4], advanced Hodgkin's disease and non-Hodgkin's lymphoma

Table 3. Inactivation of NMN adenylyltransferase from human placenta by *C*- and *N*-nitroso compounds*

Effector (1.3 mM)	Relative enzyme activity: Forward reaction (NAD synthesis) 0′	20′	Reverse reaction (NAD pyrophosphorolysis) 0′	20′
None	100	100	100	100
BCNU	100	29	100	32
Nitrosobenzene	69	3	77	8
MNNG	80	71	100	80

* Enzyme activities were tested upon 0–20 min incubation of the enzyme with the selected compound at 37°. Conditions were: 70 mM Tris buffer, 28 mM $MgCl_2$, pH 7.5. At 0′ and 20′ samples were withdrawn from the incubation mixture and the residual activity expressed as per cent fraction of reference sample activity. Enzyme activity assay was performed by HPLC quantitation of the reaction products and substrates, as described in Materials and Methods.

[5, 6, 10]. Recently BCNU has been used, in combination with other drugs, in chemo/hormonal therapy of metastatic melanoma [11].

Despite data that relate the effect of BCNU to its ability to interfere with DNA synthesis, DNA repair, and other enzymatic systems involved in crucial cellular events [12–14], the detailed mechanism is still unknown; in particular the reasons for the preferential toxicity of BCNU for neoplastic tissues are not understood.

Rice *et al.* [2], as reported above, ascribe the selective killing of human malignant cells caused by nitroso compounds to the inactivation of the enzyme poly(ADP-ribose) polymerase. This finding does not exclude *a priori* the possibility that these compounds could affect other important metabolic processes. In this report we have examined the effect of BCNU and other *C*- and *N*-nitroso compounds on human NMNAT, the enzyme involved in the final step of biosynthetic pathway leading to pyridine dinucleotide formation. We found that BCNU and nitrosobenzene significantly depress the enzymatic activity of NMNAT, while MNNG appears to be less effective. Our data indicate that the loss of activity is dependent on incubation time, temperature and nitroso compound concentration. Furthermore the protective effect exerted by DTT and β-mercaptoethanol strongly suggests that an essential reason for the inactivation caused by BCNU could be the oxidation of NMNAT thiol groups.

Thus, the results obtained reveal adverse effects of BCNU and other nitroso compounds on the human enzyme involved in NAD biosynthesis; even though these data do not disqualify poly(ADP-ribose) polymerase inactivation as a key component in the cellular response to such compounds, changes in cellular processes attributed to reduced poly(ADP-ribose) concentrations might also be due to the compromised integrity of the metabolic pathway leading to NAD synthesis.

In this regard it is important to explore the behavior of NMNAT with respect to BCNU inactivation, both in normal and tumor cells; a knowledge of this behavior could also be very helpful in chemotherapy strategy.

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