Effects of the Antitumor Agent 8-Carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one on the DNA of Mouse L1210 Cells

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ABSTRACT

L1210 murine leukemia cells were treated in vitro with the novel antineoplastic agent 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (M&B 39565), and its interaction with cellular DNA was assessed by alkaline elution. DNA interstrand cross-link and DNA-protein cross-link formation was quantified with regard to drug concentration and length of incubation time after a 2-hr incubation period with drug. Cytotoxicity, as measured by colony formation assays, and DNA damage caused by M&B 39565 were compared with those caused by a breakdown product of M&B 39565, 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (MCTIC) and also with 1-(2-chloroethyl)-1-nitrosourea (CNU). Both MCTIC and CNU decompose to yield a 2-chloroethylidazole species which is capable of alkylating DNA. At equimolar concentrations, all three drugs possessed similar in vitro cytotoxicities; at equitoxic concentrations, they produced similar levels of DNA interstrand cross-linking. The time course for cross-link formation was different for CNU when compared with MCTIC and M&B 39565, with peaks at 6 hr (CNU) and 9 hr (M&B 39565 and MCTIC). This study suggests that M&B 39565 is cytotoxic against L1210 leukemia cells as a consequence of DNA interstrand cross-link formation, probably via its breakdown product MCTIC.

INTRODUCTION

M&B 39565 (Chart 1) is a novel synthetic heterocycle (14) which possesses potent and broad-spectrum activity against murine tumors in vivo, effecting cures of the L1210 and P388 leukemias, the Lewis lung and colon 38 carcinoma, TLX5 lymphoma, and P815 A plasmacytoma (7). It also has activity against the lung LX1 human tumor xenograft, and Phase II studies have been commenced recently in humans. Further details of the biological activity of the drug will be described elsewhere.

The impetus for the screening of M&B 39565 in part arose from our study of the mechanism of action of the haloalkyl nitrosoureas (5) where we had found that the murine TLX5 lymphoma, but not the L1210 leukemia, appeared sensitive to the haloalkyl nitrosoureas because of the release of isocyanate moieties. M&B 39565 is not a nitrosourea but appeared to have, chemically, the potential to release an isocyanate (chloroethylisocyanate) (14). We thus hypothesized that it should be selectively cytotoxic to the TLX5 lymphoma but not to the L1210 leukemia. However, its broad spectrum of activity, as listed above, and analysis of breakdown products in vitro show that M&B 39565 instead decomposes via nucleophilic attack at C-4 (Chart 2) to yield the chloroethyltriazenie MCTIC (Chart 2) and not chloroethylisocyanate (14). Acid-catalyzed breakdown of MCTIC may give rise to the same chloroethylidazo species which arises from the base-catalyzed breakdown of haloethylnitrosoureas such as CNU (Chart 2) (13, 15).

The pharmacological activity of the antineoplastic haloethylnitrosoureas has been attributed to their ability to form interstrand cross-links in DNA which consist of an ethylene bridge between 2 nucleophilic sites (9). In this study, we have compared the DNA damage caused by M&B 39565, MCTIC, and CNU in L1210 cells as measured by alkaline elution (10), and we have attempted to relate this DNA damage to the cytotoxic actions of these 3 drugs in vitro.

MATERIALS AND METHODS

Cell Culture. Mouse L1210 leukemia cells were grown in spinner culture in RPMI 1630 medium supplemented with 20% heat-inactivated (56°, 30 min) fetal calf serum (Dutchland Laboratories, Denver, PA), 1 mm l-glutamine, penicillin, and streptomycin. Stock cultures were maintained in exponential phase at a density of 0.3 to 1.8 x 106 cells/ml. Colony-forming ability was determined by the soft-agar technique described by Chu and Fisher (3). Following a 2-hr drug treatment at 37° in RPMI 1630 medium, cells were seeded into polystyrene culture tubes (Falcon Plastics, Oxnard, CA) containing RPMI 1630 medium and 0.1% agar (Difco Laboratories, Detroit, MI). The efficiency of untreated control cells in forming colonies was approximately 80% in this system.

The DNA of L1210 cells used in alkaline elution assays was radioactively labeled by growing 3 x 105 cells for 24 hr in either [14C]thymidine, 0.02 μCi/ml (=56 mCi/mmol; New England Nuclear, Boston, MA), or [3H]thymidine, 0.05 μCi/ml (20 Ci/mmol; New England Nuclear) and 10–6 M unlabeled thymidine.

Drug Treatment. M&B 39565 and MCTIC were synthesized by Dr. R. Stone and Dr. M. F. G. Stevens, Department of Pharmacy, University of Aston, Birmingham, England (14). CNU was obtained from the Drug Development Branch, National Cancer Institute. Each drug was dissolved in sterile dimethyl sulfoxide immediately before treatment of cell cultures. Cells (1 x 10⁶/ml) were treated with various concentrations of drug for 2 hr at 37°. The concentration of dimethyl sulfoxide in either control or treated cells was never greater than 2% (v/v). Following drug exposure, the cells were washed 3 times by centrifugation in RPMI 1630 medium supplemented with 20% fetal calf serum at 400 x g and resuspension in fresh medium. Following this wash, cells were either assayed immediately for DNA damage by alkaline elution or incubated at 37° for various periods of time before assay.

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The abbreviations used are: M&B 39565, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (also designated NSC 353451, CCRG 81010; MCTIC, 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide; CNU, 1-(2-chloroethyl)-1-nitrosourea; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cylohexyl-1-nitrosourea.

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Assay of DNA Damage by Alkaline Elution. The basic principles involved in the detection of DNA damage by the alkaline elution assay have been published (10), and the methodology has recently been reviewed in detail (11). In this study, alkaline elution, with and without the proteinase K modification, was utilized to quantify a variety of drug-induced DNA lesions.

Assay of DNA-Protein Cross-Links. The assay of drug-induced DNA-protein cross-links utilizes the protein-binding properties of polyvinyl chloride filters. At various times after drug treatment, cells were irradiated at 0° with 300 R γ-irradiation from a 137Cs source (dose rate, 314 R/min). After suspension in phosphate-buffered saline, cells were gently layered onto 0.8-μm-pore size polycarbonate filters (Nuclepore, Pleasanton, CA) and immediately after layering, the cells were lysed as described in the DNA-protein cross-link assay. Following lysis, 2 ml of 2% sodium dodecyl sulfate-0.02 m EDTA-0.1 m glycine (pH 10.0), containing 0.5 mg/ml proteinase K (EM Laboratories, Darmstadt, West Germany), were added to a reservoir over the polycarbonate filters and pumped through the filter for approximately 1 hr at 2 ml/hr. DNA was eluted from the filters by pumping 0.02 M EDTA adjusted to pH 12.1 with tetrapropylammonium hydroxide, which contained 0.1% sodium dodecyl sulfate. Filters and fractions were processed as before (11).

DNA interstrand cross-linking indices were calculated from the formula:

\[
\text{Cross-link index} = \sqrt{\frac{1 - r}{1 - r_0}} - 1
\]

where \( r \) is the retention of drug-treated and irradiated DNA and \( r_0 \) is the 300-R-irradiated control retention (11).

RESULTS

The results of the colony formation assays for cytotoxicity of M&B 39565, MCTIC, and CNU to L1210 cells are shown in Chart 3. Concentrations of 12.5, 25, and 50 μM for each drug were used subsequently to study their effects upon DNA in the alkaline elution assay. These concentrations represent approximately a 1, 2, and >3 log cell kill. The results of the assay by alkaline elution of DNA interstrand cross-links caused by incubation with equimolar concentrations of the drugs are shown in Chart 4 and represent the profiles obtained when cross-linking was maximal with respect to time. It should be noted from Chart 4 that, upon drug-free incubation posttreatment, negligible single-strand breaks were observed at either 6 or 9 hr after drug removal. Chart 5 shows the rate of accumulation of DNA interstrand...
cross-links with time and demonstrates that 50 μM M&B 39565 and MCTIC require 9 hr of posttreatment drug-free incubation before maximal interstrand cross-links were formed, whereas with CNU it took 6 hr. The time of peak cross-linking at concentrations less than 50 μM was less well defined but appeared to follow the same trend. Chart 5 also demonstrates that the quantity of DNA interstrand cross-links formed was dependent upon the concentration of drug. Chart 6 shows this to be a linear relationship when plotted as maximal DNA interstrand cross-link index against drug concentration. Similarly, a linear relationship was observed when log cell kill (Chart 3) was plotted against DNA cross-link index (Chart 6) over an identical range of drug concentrations (Chart 7). At equitoxic concentrations, it can be seen that all 3 drugs, M&B 39565, MCTIC, and CNU, produce almost identical amounts of DNA interstrand cross-links.

The kinetics of formation and removal of DNA-protein cross-links is shown in Chart 8; again, at equimolar concentrations, all 3 drugs produced similar elution profiles.

**DISCUSSION**

M&B 39565 has been found to possess potent antitumor effects in vivo when tested in murine systems, and this potency and its spectrum of activity closely resemble those of the nitroso ureas (4). One of the hypotheses leading to the screening of the drug concerned the potential release of an isocyanate from M&B 39565 (4). In fact, both chemical and biological studies have shown that no isocyanate release occurs under physiological conditions (8, 14) but instead that M&B 39565 breaks down to form the monochloroethyltriazene MCTIC (Chart 2). In the present study, a comparison of the effects of M&B 39565 with MCTIC and CNU has shown them to possess almost identical cytotoxicity to L1210 cells in vitro (Chart 3) and similar profiles of interaction with DNA (Charts 4 and 5). MCTIC and CNU may both decompose to form a 2-chloroethylidiazonium species (Chart 2), the former by an acid-catalyzed pathway (13) and the latter by base catalysis (15). The data shown here demonstrating their interaction with DNA support the hypothesis that both CNU and MCTIC, and thus M&B 39565, may act through a common reactive species to form DNA interstrand and DNA-protein cross-links. In the following paper (6), the nature of these cross-links will be addressed in greater detail.

Monochloroethyltriazenes, like MCTIC, have been the subject of a previous study of interaction with DNA (12). It was claimed that no interstrand cross-links formed when PM2-CCC DNA was treated with millimolar concentrations of chloroethyltriazines and the DNA studied by ethidium fluorescence. In our study, using alkaline elution, micromolar concentrations of MCTIC formed both DNA interstrand and DNA-protein cross-links but, unlike Lown and Singh who looked at effects immediately after an 90-min drug treatment, we measured these effects after much...
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Chart 6. Plot of DNA interstrand cross-link index against concentration at time of maximal cross-linking for each drug. Drug treatments as indicated. For M&B 39565 and MCTIC, t = 9 hr; for CNU, t = 6 hr.

Chart 7. Plot of DNA interstrand cross-link index against log cell kill at time of maximal cross-linking for each drug. Drug treatments as indicated.

longer time periods of drug-free incubation. In fact, in our studies, negligible cross-linking was observed immediately after drug treatment as compared to the maximum cross-linking levels observed at 9 hr posttreatment. The 2-step alkylation of DNA to form interstrand cross-links by the nitrosoureas is considered to involve a fast alkylation by the diazonium species at C-1 of the chloroethyl group of the released chloroethyl diazo species followed by slower loss of chloride ion at C-2 to form a 2-carbon bridge cross-link (9). Thus, our study of MCTIC reaction with L1210 cellular DNA, which monitored the slow increase in cross-links from almost zero levels immediately after treatment to a peak 9 hr posttreatment, is perhaps more compatible with what would be expected for a process closely resembling nitrosourea breakdown and alkylation of the DNA double helix.

The direct relationship shown here (Chart 7) between the cytotoxicity of M&B 39565 and the quantity of DNA interstrand cross-links formed strongly suggests that the mechanism of action of this drug is related to its DNA-damaging effects in L1210 cells. The close similarity of these effects to those produced by the nitrosourea CNU may explain the patterns of cross-resistance observed between M&B 39565 and nitrosoureas in murine tumors (4). The nitrosoureas most commonly used in the clinic have proved to be somewhat disappointing because of their toxicity to the bone marrow (2). The potential of nitrosoureas, such as BCNU and CCNU, to carbamoylate biological targets has been implicated in this toxicity (1). In this respect, M&B 39565 may present a considerable advantage over these compounds because, it is shown here to have equivalent reaction with DNA as a nitrosourea but does not carbamoylate enzymatic systems (8). As a latentiated form of the monochloroethyltriazene MCTIC, M&B 39565 has biological properties similar to those of this compound, e.g., in its effects on growth of the L1210 tumor in vivo (13, 14). However, unlike MCTIC, which is a notoriously labile compound, particularly in acidic media (13), M&B 39565 has a half-life of 98 min in phosphate buffer, pH 7.4 (14). These characteristics will hopefully allow M&B 39565 to become a useful drug in the treatment of human cancers. The studies reported in the following paper suggest that some human tumor cells may indeed be more sensitive to this drug than to a number of existing compounds currently used in the clinic, such as BCNU or CCNU.

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