DNA Cross-Linking and Cytotoxicity in Normal and Transformed Human Cells Treated in Vitro with 8-Carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

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ABSTRACT

Normal (IMR-90) and SV40-transformed (VA-13) human embryo cells were treated with 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (M&B 39565), and the effects of the drug on cell viability and cellular DNA integrity were studied. The effects of M&B 39565 were compared with one of its potential decomposition products 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (MCTIC). M&B 39565 and MCTIC were 5- to 6-fold more toxic to VA-13 cells than to IMR-90 cells for drug concentrations which produced a 2-log cell kill, as measured by colony-forming assays. Using alkaline elution analysis, VA-13 cells exhibited concentration-dependent DNA interstrand cross-link formation. In IMR-90 cells, little or no interstrand cross-link formation was detected. The DNA interstrand cross-link formation in VA-13 cells was found to peak 12 hr after drug removal. A linear correlation between DNA interstrand cross-link formation and log cell kill was observed in VA-13 cells but not in IMR-90 cells. DNA-protein cross-link formation was found to be comparable in both cell lines for each drug, suggesting that drug penetration and intracellular drug reactivity were similar. Initial chemical decomposition studies suggest that both M&B 39565 and MCTIC may produce a chloroethyldiazido species. This species has been implicated in the formation of chloroethyl-DNA adducts which convert to DNA interstrand cross-links in mammalian cells treated with chloroethylnitrosoureas [Erickson et al., Nature (Lond.), 288: 727, 1980]. These data suggest that DNA interstrand cross-link formation may be a common mechanism for the in vitro cytotoxicity of M&B 39565 and MCTIC.

INTRODUCTION

M&B 39565 is a novel bicyclic heterocycle which demonstrates a broad spectrum of antitumor activity against murine tumors (5) and is currently undergoing a Phase II trial in humans. M&B 39565 appears to be a prodrug of the monochloroethyltriazene MCTIC. In the preceding paper (4), we studied the effects of MCTIC, M&B 39565, and the nitrosourea CNU on viability of the L1210 leukemia in vitro and on L1210 cellular DNA as assayed by the alkaline elution technique. All 3 compounds had similar cytotoxicity, on a molar basis, and at equitoxic concentrations they produced similar levels of DNA interstrand cross-links. This close equivalence of DNA damage produced by CNU and M&B 39565 is perhaps not surprising given that both may decompose to give a chloroethyldiazido species (9–11, 13). This putative chloroethyldiazido species has been considered to be responsible for the formation of monofunctional adducts in DNA and for the consequent formation of DNA interstrand cross-links, which are formed as halide loss occurs from a chloroethyalted base on one of the DNA strands (7).

In a previous study of nitrosourea cytotoxicity, Erickson et al. (2) showed that normal (IMR-90) and SV40-transformed (VA-13) human embryo cells differed in their response to chloroethylnitrosoureas, possibly as a consequence of their capability to repair O6-guanine lesions. These cells have been classified as either O6-methylguanine repair proficient (Mer+, IMR-90) or deficient (Mer−, VA-13), and it was suggested that the methylation repair capability may extend to a capability to repair O6-chloroethylnitrosourea monoadducts. The removal of such an O6-chloroethylnitrosourea monoaadducts, by a Mer+ cell, would prevent consequent cross-linking and enhance the chance of survival of the cells. Furthermore, Erickson et al. (3) have shown that chloroethylnitrosoureas are less toxic to a wide range of cells with the Mer− phenotype when compared with those of the Mer+ phenotype. Thus, such a difference in repair capacity may then explain the preferential cytotoxicity of chloroethylnitrosoureas to Mer− cells, as well as to the VA-13 cell line.

We report here measurements of the cytotoxicity of M&B 39565 and the product of its decomposition, MCTIC, to VA-13 (Mer−) and IMR-90 (Mer+) cells. The technique of alkaline elution has been used to quantify DNA damage, particularly DNA interstrand cross-links, in both cell types, and the relationship between cytotoxicity and the formation of cross-links has been assessed.

MATERIALS AND METHODS

Cell Proliferation Assay. Both IMR-90 and VA-13 cells were seeded at 2 × 104 cells/25-cm2 flask and incubated for 24 hr. The cells were then treated with various concentrations of drug for 2 hr at 37°C in MEM (Dutch: Laboratories, Denver, PA), supplemented with 10% fetal bovine serum, gentamycin (0.05 mg/ml), glutamine (0.3 mg/ml), B6, biotin (0.1 mg/ml), vitamin B12 (1.36 µg/ml), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.02 µM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Following drug exposure, the medium was removed by aspiration, and fresh MEM was added. After control cells had completed at least 3 population doublings, cells were harvested by trypsinization with a solution of 0.05% trypsin in Ca2+, and Mg2+-free Hanks' balanced salt solution containing 0.02% EDTA and counted in an electronic cell counter (Model ZBI; Coulter Electronics, Hialeah, FL). Inhibition of cell
proliferation in treated cultures was compared to the cell proliferation in untreated control cultures.

Colonies Formation Assays. Both IMR-90 and VA-13 cells were seeded at 0.1, 0.3, 1, and 3 x 10^5 cells/50-mm-diameter plastic dish (Falcon film-lined dish; Becton-Dickinson, Oxnard, CA) in MEM. Cells were preincubated for 24 hr to allow attachment to the surface of the dish and then exposed to various concentrations of either M&B 39565 or MCTIC for 2 hr at 37°. After 10 days of incubation in fresh MEM, the plates were rinsed with Hanks' balanced salt solution, fixed with methanol, and then stained with a solution containing 1 ml methylene blue, 1 ml 0.15 M Na2HPO4, and 1 ml 0.15 M KH2PO4 diluted to 50 ml with distilled water. Colonies were counted. The observed control plating efficiencies were 41% for IMR-90 cells and 76% for VA-13 cells.

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 (11).

Each drug was dissolved in sterile dimethyl sulfoxide immediately before treatment of cell cultures. The concentration of dimethyl sulfoxide in either treated or control cells was never greater than 2% (v/v). Treatments were terminated by aspiration of the drug-containing medium before treatment of cell cultures. The concentration of dimethyl sulfoxide in either treated or control cells was never greater than 2% (v/v). Treatments were terminated by aspiration of the drug-containing medium and replacement with fresh MEM.

Cell Culture for Alkaline Elution Assays. IMR-90, a human fibroblast cell line derived from embryonic lung, was obtained from W. Nichols, Institute of Medical Research (Camden, NJ). VA-13 cell line was derived by transformation of the normal human embryonic cell strain WI-38 with SV40 and has been maintained in this laboratory for more than 5 years.

Stock cultures of both IMR-90 and VA-13 cell lines were maintained by seeding cells at a density of 5 x 10^5 cells/ml, at 37°, in Eagle's MEM supplemented as described above. For alkaline elution assays, 1.5 to 2.5 x 10^5 cells were seeded into 25-cm^2 flasks in 10 ml MEM which contained [14C]thymidine, 0.02 μCi/ml (>56 mCi/mmol; New England Nuclear, Boston, MA), and were grown for 24 hr. The radioactive medium was removed, and the cultures were grown for an additional 18 to 24 hr to allow for the maturation of labeled DNA into high-molecular-weight DNA. L1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium supplemented with 15% heat-inactivated (56°, 30 min) fetal calf serum.

The DNA of L1210 cells was labeled by growing 3 x 10^5 cells/ml for 24 hr in RPMI 1630 medium which contained [3H]thymidine, 0.05 μCi/ml (20 Ci/mmol; New England Nuclear), and 10^-6 M unlabeled thymidine.

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 (8), and the methodology has recently been reviewed in detail (9). In this study, the technique of alkaline elution was varied in order to quantify a variety of drug-induced DNA lesions as described in the preceding paper (4).

RESULTS

Assays of the cytotoxicity of M&B 39565 to IMR-90 and VA-13 cells are shown in Chart 1. Inhibition of cell proliferation of VA-13 cells was 7-fold greater than for IMR-90 cells when compared at a 50% inhibition level. This differential cytotoxicity was also observed in a colony-forming assay, and Chart 2 shows these results for both M&B 39565 and its decomposition product MCTIC. In this case, inhibition of the colony-forming ability of VA-13 cells was 6-fold greater than that of IMR-90 cells for M&B 39565 and 5-fold greater for MCTIC when cell kill was >1 log.

Assays of DNA damage were performed on both cell lines, VA-13 and IMR-90, treated with M&B 39565 or MCTIC, and the elution profiles are shown in Chart 3. These represent the profiles of control cultures treated with 300 rads alone, in which case, after the formation of random single-strand DNA breaks, the elution profile is first order with respect to time. In addition, the profiles of VA-13 (Mer-) or IMR-90 (Mer+) cells treated with M&B 39565 or MCTIC and 300 R are shown. The time course of the formation of DNA interstrand cross-links in the VA-13 cell line is shown in Chart 4 and peak levels were obtained 12 hr posttreatment for both drugs. It is at this time of maximal cross-link formation that the profiles of Chart 3 are shown. Concentrations of both M&B 39565 and MCTIC which give a >1.5-log cell kill (Chart 1) produced insignificant levels of DNA interstrand cross-links in the IMR-90 cell line (Charts 3 and 4). Calculation of DNA interstrand cross-link index is seen to be linear for both M&B 39565 and MCTIC in the VA-13 (Mer-) line, but insignificant amounts of DNA damage occur in the IMR-90 (Mer+) cell line. When a 2.5-log cell kill of IMR-90 cells by MCTIC or a 1.5-log cell kill by M&B 39565 occurs, cell death may be the consequence of something
other than DNA interstrand cross-links (see Chart 6).

One possible explanation for the differential in both cytotoxicity and DNA interstrand cross-link formation between VA-13 and IMR-90 cells would be that drug uptake is different in the 2 cell lines. Table 1 shows that the formation of DNA-protein cross-links is of an equivalent order in both Mer- and Mer+ cell lines and thus that drug penetration and reaction with a chromatin-associated target is equivalent.

DISCUSSION

Studies on the effect of haloalkynitrosoureas on cells which were either capable or not of repairing the O6-methylguanine lesion in their DNA suggested that the nitrosoureas may form Table 1

DNA-protein cross-link indices in VA-13 and IMR-90 cell lines
Values are the means of at least 3 experiments.

<table>
<thead>
<tr>
<th>Cross-link index</th>
<th>Concentration (µM)</th>
<th>0 hr after drug treatment</th>
<th>6 hr after drug treatment</th>
<th>12 hr after drug treatment</th>
</tr>
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<tbody>
<tr>
<td>M&amp;B 39565</td>
<td>25 -0.002 -0.002</td>
<td>-0.04 0.023</td>
<td>0.032 0.030</td>
<td>0.079 0.097</td>
</tr>
<tr>
<td></td>
<td>50 0.003 0.0001</td>
<td>0.025 0.015</td>
<td>0.049 0.064</td>
<td>0.079 0.097</td>
</tr>
<tr>
<td></td>
<td>100 -0.003 0.002</td>
<td>0.040 0.064</td>
<td>0.082 0.072</td>
<td>0.079 0.097</td>
</tr>
<tr>
<td></td>
<td>150 0.045 0.009</td>
<td>0.082 0.072</td>
<td>0.079 0.097</td>
<td>0.079 0.097</td>
</tr>
<tr>
<td>MCTIC</td>
<td>25 -0.002 0.006</td>
<td>0.004 0.019</td>
<td>0.029 0.020</td>
<td>0.053 0.066</td>
</tr>
<tr>
<td></td>
<td>50 0.019 0.010</td>
<td>0.016 0.021</td>
<td>0.053 0.066</td>
<td>0.053 0.066</td>
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<tr>
<td></td>
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<td>0.050 0.038</td>
<td>0.053 0.066</td>
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<tr>
<td></td>
<td>150 0.109 0.095</td>
<td>0.093 0.088</td>
<td>0.053 0.066</td>
<td>0.053 0.066</td>
</tr>
</tbody>
</table>
Mechanism of Action of M&B 39565 on Human Cells

haloethyl adducts which may be repaired in a way similar to that for the methyl adduct (3, 14). Thus, it would appear that an initial lesion formed in DNA upon the decomposition of nitrosoureas to a halomethylazole species may be an O6-guanine adduct. A second reaction involving the slow cross-linking of the DNA then occurs. Repair of the initial lesion at O6-guanine by a Mer+ cell would therefore prevent the expression of interstrand cross-links and also apparently much of the cytotoxicity (14). In this study with M&B 39565, a similar series of findings has been presented, namely, that Mer- cells (VA-13) are more sensitive to M&B 39565 than are Mer+ cells (IMR-90) (Charts 1 and 2), with a linear relationship between cytotoxicity and the number of interstrand cross-links formed in the VA-13 cells (Chart 6). In the Mer+ cell line, IMR-90, M&B 39565 both was less cytotoxic and formed negligible DNA interstrand cross-links. A differential cytotoxicity between IMR-90 and VA-13 greater than that seen with the clinically used nitrosoureas CCNU and BCNU was observed (2). This may signify that M&B 39565 forms more O6-guanine adducts than do either CCNU or BCNU in the VA-13 cell line. On the other hand, in the L1210 cell line, equivalent cytotoxicity and cross-linking was observed between CNU and M&B 39565, the only difference between them being the rate of cross-link formation. This time difference may provide a partial explanation for the larger differential seen here for M&B 39565 when compared with BCNU (2), namely, that the slower rate of reaction allows a greater repair capacity in the Mer+ cells where the protein turnover is apparently required for repair capacity to be continued (12). The other relevant factor is that, unlike BCNU or CCNU which are capable of carbamoylating cellular targets, one of which may be a DNA polymerase (1), M&B 39565 has no carbamoylating activity (6). The inactivation of a DNA polymerase or of any aspect of repair function will obviously compromise a Mer+ cell. Thus, M&B 39565 may present considerable advantages in the clinic, where carbamoylation arising from the nitrosoureas BCNU and CCNU has been implicated, but not proven, to reduce their chemotherapeutic index.

M&B 39565 decomposes to the monochloroethyltriazene MCTIC (11). As with our previous study comparing both cytotoxicity and DNA damage in L1210 cells after treatment with M&B 39565 and MCTIC (4), the 2 drugs demonstrate comparable cytotoxicity and DNA damage. MCTIC was found to be marginally more cytotoxic to IMR-90 cells than was M&B 39565, although neither compound caused significant DNA interstrand cross-links in this cell line.

In conclusion, the present studies suggest that the potent and novel heterocycle M&B 39565 is cytotoxic against the transformed cell line by virtue of its capability to form DNA interstrand cross-links which probably arise after an initial alklylation at the O6 position of guanine residues in DNA. The lack of carbamoylation arising from M&B 39565, in comparison with clinically used nitrosoureas capable of forming the same DNA adducts, may be one possible reason for the greater differential cytotoxicity between Mer+ and Mer- cells (2). It is to be hoped that such a differential will be maintained in the clinic between host and tumor, allowing M&B 39565 to be a useful agent in the treatment of cancers.

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