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Scheduling effects of 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) and 1,3-Bis-chloroethyl-1-nitrosourea (BCNU) on mouse melanoma cells B16CL5

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ABSTRACT

Title of Thesis: Scheduling Effects of 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) and 1,3-Bis-chloroethyl-1-nitrosourea (BCNU) on Mouse Melanoma Cells B16CL4

Gracie V. Coffey, Master of Science, Environmental Engineering, Designated Degree, (Toxicology Option), 1985

Thesis directed by: Professor Helene Z. Hill and Professor Richard Trattner

DTIC and BCNU have been demonstrated to have enhanced chemotherapeutic effects in mice bearing B16 mouse melanoma cells. The order of administration seems to be important in maintaining optimum effects. This was shown is a study by Hill et al. (unpublished).

In the present study, mouse melanoma cells, B16CL4, were treated with either DTIC or BCNU. The drug was allowed to remain on the cells for 2, 3, or 4 days. The drug-containing media were then removed, the cells were washed and fresh medium was added. The cells were incubated in an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C. In combination experiments, dishes of B16CL4 cells were treated with DTIC on day 0. BCNU was then added on days 0, 1, or 2 of exposure. At the end of the allotted time the drug-containing media were removed and replaced with fresh medium. The dishes were incubated under the same conditions as those used for the controls. This procedure was repeated except that BCNU was added first and DTIC was added on day 0, 1, or 2 of exposure. The dishes

were incubated for 15 days and the colonies were then fixed and stained. All colonies containing 50 cells or more were counted.

The results showed that DTIC alone enhanced cell death the longer it remained in contact with the cells. BCNU alone had no effect at the concentration used.

The results for the dose schedule when DTIC was added on day 0, followed by BCNU on day 2, showed a 60% decrease in the observed surviving fraction of cells from the expected surviving fraction of cells. This indicated that melanoma cell death was enhanced and these results are in agreement with the previous study conducted by Hill et al. (unpublished).

The results for the dose schedule when BCNU was added on day 0, followed by DTIC on day 1, showed a 66% decrease in the observed surviving fraction of cells from the expected surviving fraction of cells. But then, the decrease in observed surviving fraction of cells from the expected surviving fraction of cells rose from 66% to 26%. These results were similar to the in vivo results seen in the study conducted by Hill et al. (21). However, these in vivo effects were not as dramatic as the effects seen when DTIC was added first.

SCHEDULING EFFECTS OF 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) AND 1,3-Bis-chloroethyl-1-nitrosourea (BCNU) ON MOUSE MELANOMA CELLS B16CL4

by
Gracie V. Coffey

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Master of Science, Environmental Engineering Designated Degree (Toxicology Option) 1985

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DEDICATION

I wish to dedicate this to my father, Mr. Leonard J. Verrilli Jr. for his support and love throughout the years. I would also like to thank my other family and friends, Jerry, Corien, Barry, Sue, Ian, Leslie, Mel, Marie, Anne, and Lenny for their love and support.

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TABLE OF CONTENTS

LIST OF TABLES

I. INTRODUCTION

BCNU has many distinctive properties.

1). It is very unstable and will decompose nonemzymatically to yield vinyl carbonium ions, chloroethyl carbonium ions and organic chloroethyl isocyanates. The chloroethyl carbonium ion (or chloroethyldiazonium precursor) comes from the nitroso moiety of the parent compound (24). Further decomposition of this ion generates volatile products, such as chloroethanol, acetaldehyde, vinyl chloride and dichloroethane (11). It is this ion that is considered to be responsible for the alkylating effects with nucleic acids that are observed in biological systems.

The chloroethyl isocyanates are derived from the N-3 moiety of the parent compound (24). The isocyanate causes carbamoylation reactions with electron rich groups like the amino groups of proteins which generate stable urea derivatives (7).

2). BCNU is very reactive and its chemical and biological half-life is approximately 15 to 30 minutes (39).

3). It is lipid soluble and will readily cross the blood-brain barrier. It is also easily transported into living cells (46).

4). BCNU displays bone marrow toxicity in man (30).

5). BCNU is equally effective, for a given concentration, on

the plateau phase of growth and on cycling or actively dividing cells (3).

6). The metabolic products of BCNU will bind to macromolecules by two mechanisms, alkylation and carbamoylation (39).

The two prominent products of nitrosourea decomposition are isocyanates and alkyldiazohydroxides. The latter compounds are capable of alkylating nucleic acids and are considered to be responsible for the antitumor effects observed in biological systems (46).

DNA cross-links are usually produced by bifunctional alkylating agents which cause bridge formations usually at guanine N-7. Because this moiety of BCNU is only capable of monofunctional alkylation, there was some question as to the mechanism involved in the cytotoxicity of this agent. However, studies have shown that the chloroethylnitrosoureas are capable of cross-linking DNA, even though they have only one alkylating functional group (26).

The cross-linking mechanism occurs in two steps. It starts with the chlorethylation of a nucleophilic site on one of the DNA strands. Then the reaction continues with the displacement of Cl^- by a nucleophilic site on the opposite strand. This results in the formation of an ethyl bridge between the two strands. Thus, BCNU can react with nucleic acids causing structural changes (26).

There is still some question as to the sites of reaction of BCNU with DNA. Kramer et al. (28) identified two products of the reaction of BCNU with polycytidylic acid. The first product, 3-hydroxyethyl-CMP, is formed by the initial chloroethylation of cytosine, followed by a displacement of Cl^- through a hydrolysis reaction.

The second product, 3,N^4-ethano-CMP, is also formed by the initial chloroethylation of cytosine, but the displacement of Cl^- in this case is caused by a second alkylation of the cytosine moiety (28). It has become evident that BCNU can chloroethylate many different DNA sites. It is also possible that a second alkylation occurs at another DNA site along with the displacement of Cl^-.

Some problems arise when trying to determine the sites where these reactions are most likely to occur. In order for an interstrand cross-link to be formed, the reaction sites must be on opposite strands of DNA. But to complete the cross-link, the two opposed reaction sites must be spanned by a bridge which is composed of only two carbon atoms. This limits the possible sites where cross-linking can occur, unless the helix is severely distorted by the chloroethylation reaction. The sites involved must be the ones that are usually involved in hydrogen bonding between the two bases. One pair of sites has been suggested by Kohn (26) as involving a bridge that is formed across the guanine

O-6 and the cytosine N-4. There has been some evidence to support this hypothesis. Kramer et al. (28) found that the cytosine N-4 position was involved in an intramolecular cross-linking when polycytidylic acid was reacted with BCNU. In another study by Singer, et al. (48) it was found that ethyl-nitrosourea ethylated the guanine O-6 of DNA.

In another study BCNU was shown to react with two synthetic polynucleotides. This compound is not considered a typical bifunctional alkylating agent, but it does possess some ability to alkylate polycytidylic and polyuridylic acid. Ludlum et al. (33) attempted to determine the structural modifications induced by BCNU. He isolated three nucleotide derivatives, 3-(β-hydroxyethyl)-CMP, 3,N^4-ethano-CMP, and 7-(β-hydroxyethyl)-GMP. All of these altered bases were created by the generation of a two-carbon fragment which is found in the chloroethyl carbonium ion. This two-carbon unit is free to interact with the nucleotides and causes the changes observed in this study.

There are, however, other studies that do not show any direct evidence which supports the findings mentioned in the preceding studies. The previous evidence suggested that the alkylation mechanism, and not the carbamoylation mechanism, caused by BCNU is the mechanism that will inhibit DNA synthesis in certain cases. Wheeler et al. (53) found that using BCNU concentrations of 2.5×10^{-3} M caused a decrease

in DNA synthesis by intact L1210 cells and also a decrease in the activity of the DNA polymerase enzyme, nucleotidyl-transferase. However, this concentration of BCNU did not change the DNA primer activity. Yet a concentration of 1×10^{-3} M of BCNU inhibited the synthesis of DNA, but without a decrease in the DNA nucleotidyltransferase activity. Therefore, the deactivation of this enzyme is probably not the cause of decreased DNA synthesis. During this study, it became evident that when a substituent was present on the N-3 of the nitrosourea, the activity of the nitrosourea was increased. Thus, the newly generated isocyanate (derived from the decomposition of the parent compound) could possibly be involved in the inhibitory activity of DNA synthesis. This is consistent with the findings that BCNU and 2-chloroethyl isocyanate were equal in inhibitory activity in this study. Also, it appeared that the inhibition of the incorporation of ^{14}C from deoxythymidine-5'triphosphate-2-^{14}C into nucleic acids by the enzyme preparations from L1210 ascites cells was caused by the 2-chloroethyl isocyanate, and not the alkylating moiety that is generated when the BCNU molecule is cleaved. Thus, it was concluded that the carbamoylation of the polymerase enzyme, and not its alkylation, is responsible for the inhibitory activity of BCNU.

The isocyanates, which are very reactive and have strong

carbamoylating activity, are not considered to play a crucial role in the antineoplastic effects of the parent nitrosoureas. However, the activity of the isocynates is of significance because they can alter proteins and inhibit DNA repair processes. This repair mechanism could be responsible for preventing cells from being killed by oncolytic compounds which cause DNA damage. There has been some evidence to support this hypothesis.

Kann et al. (23) showed that 2-chloroethyl isocyanate (CIS) interfered with repair of DNA damaged by x-rays or ultraviolet radiation. It was thought that this effect was caused by the inhibition of the enzymes that bind to nucleic acids. The mechanism assumed to be responsible for this effect is thought to involve the loss of a positively charged lysine amino group. This could interfere with the ability of a protein to bind to a negatively charged nucleic acid. A carbamoylation reaction is believed to cause this loss of the lysine amino group. However, there is no direct evidence demonstrating that the repair enzymes are inhibited. It is hypothesized that another mechanism could be involved such as the modification of nonenzymatic nuclear proteins.

Fornace et al. (15) continued to try to prove that CIS does interfere with repair mechanisms. It was found that CIS did not affect the endonuclease step of repair synthesis due to UV-induced strand breaks, but it did prevent the rejoining

of strand breaks. This indicated that the ligase step in DNA repair was inhibited. CIS is unique because it is the only known compound that can cause this effect on the ligase step of DNA repair.

In another study Wheeler et al. (53) showed that CIS was responsible for the decreased activity of DNA nucleotidyl-transferase when crude enzyme preparations from L1210 ascites cells were incubated with concentrations of 2.5×10^{-3} M of BCNU. Thus, under some conditions BCNU and its decomposition product, CIS, can cause a decrease in the DNA nucleotidyl-transferase system. But the deactivation of this enzyme is probably not responsible for a decrease in DNA synthesis *in vivo*.

Baril et al. (2) found that CIS affected an enzyme involved in the DNA replication process and not the repair process. CIS inhibited only one of the two nonmitochondrial DNA polymerases, DNA Polymerase II, that was purified from rat liver and hepatoma cells. Although the function of this enzyme is unclear, it is thought that it plays a role in the DNA replication of tissues. The activity of this enzyme is low in nonproliferating tissues and increases as the growth rate of the tissues increase. The DNA polymerase I was not inhibited by CIS. This enzyme does not respond when cells are proliferating and is thought to be involved with the repair processes. The mechanism of action is still an

untested assumption, but it is believed that the observed effects are caused by the carbamoylation of a critical cysteine moiety located at or near the site of the polymerase enzyme. This is thought to be true because the isocyanates are known to form stable carbamoyl derivatives with many amino acids in proteins. This mechanism is probably not the only mechanism that causes the enzyme inhibition because it was found, in this study, that a carbamoyl derivative of cysteine can be unstable at the pH used for the enzyme assay.

Many other effects observed in biological systems could be due to the carbamoylating effects of BCNU. Kann et al. (24) found that CIS was responsible for the inhibition of RNA maturation of ribosomal precursor RNA and with nucleoplasmic messenger precursor RNA. During the maturation process the high-molecular weight RNA chains are shortened. The 45 S and 32 S chains in the nucleolus were prevented from being shortened by cleavage and degradation. Thus, the inhibition caused by BCNU resulted in the persistance of long RNA molecules. In addition these long chains were prevented from leaving the nucleoplasm.

This observed effect is not the one that is hypothesized as the mechanism responsible for the therapeutic antitumor effects of BCNU. The structural activity of some of the nitrosoureas requires that the N-3 nitrogen of the urea moiety be substituted. The isocyanates of BCNU and

1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) were found to be more effective than the parent compounds. Because of this observed effect, it is thought that the metabolites are the ones responsible for the inhibition of RNA maturation. If the N-3 nitrogen substituent is necessary for the metabolite to cause the effects on RNA, then the isoccyanate product of CCNU would be inactive. This is not the case. This decomposition product is very active against neoplasms. Thus, it was concluded that RNA maturation inhibition and/or other effects caused by the isocyanates are not the effects responsible for antineoplastic activity. It is more likely that these effects cause the toxic side effects associated with the use of these drugs (24).

Another effect of the carbamoylating activity of BCNU is observed during de novo purine biosynthesis. It affects the biosynthesis process in several ways. First, it increases the rate of incorporation of formate-^{14}C into purines. BCNU is thought to affect a certain reaction which involves the insertion of either the C-2 or C-8 position of the purine ring. Second, it inhibits the contribution of histidine as a donor to the 1-carbon endogenous pool of FH_4 coenzyme derivatives which are involved in the metabolism of tetrahydrofolic acid derivatives. The metabolism of these coenzymes are part of the de novo purine nucleotide synthesis pathway. It is thus speculated that a selective

carbamoylation mechanism of these enzymes is responsible for the effects observed in this study (Groth et al. 19).

In summary, there are at least two theories that could explain the mechanisms of action of BCNU.

1). BCNU is thought to possess alkylating capabilities due to the creation of a carbonium ion which can react with nucleic acids and DNA.

2). BCNU can cause carbamoylation reactions. This effect is caused by the isocyanates which will react with electron rich groups found in the amino acids of proteins.

The characteristics of DTIC are:

1). It is very unstable and is decomposed via two mechanisms, photodecomposition and microsomal metabolism (8)(17)(31)(44).

2). The metabolites, and not the DTIC, are considered to be pharmacologically active (17)(31)(32)(44).

3). DTIC (like BCNU) appears to be non-cell cycle stage specific. Wilkoff et al. (54) proposed a first order kinetics model for the killing of L1210 cells by DTIC. Thus it was suggested that the percentage of cells killed is unrelated to the number of viable cells present.

DTIC is probably inactive in biological systems and therefore must be activated in order to produce the anti-tumor properties that have been clinically demonstrated. There are two known metabolic pathways that create bio-

logically active metabolites (17)(31)(32)(44).

The first pathway is induced by long-wave ultraviolet light which converts DTIC to 4-diazoimidazole-5-carboxamide (DZC). This metabolite is a very reactive electrophile and highly cytotoxic. Dimethylamine, a biologically non-cytotoxic compound is also created during this step of the reaction. The DZC spontaneously cyclizes to 2-azahypoxanthine (aza-Hx). This metabolite is not as toxic as its predecessor. Yamamoto et al. (55) showed that DZC was 1000 times more potent than aza-Hx as an inhibitor of E. coli cell growth. This activity of aza-Hx against E. coli was counteracted by hypoxanthine. Consequently it is doubtful whether aza-hx possesses any biological activity at all (8).

It is hypothesized that the metabolite DZC is responsible for the inhibitory effects on DNA biosynthesis (44)(51) but not on RNA or protein synthesis (55). Yamamoto (55) found that low concentrations of DZC ranging from 1 µg/ml to 10 µg/ml inhibited the growth of E. coli cells without causing the cells to lyse. This effect was also seen in resting cells, as well as in actively dividing cells. When cysteine was added to cultures incubated with DZC, the inhibition of cell growth was prevented.

Saunders et al. (43)(44) showed inhibited cell growth of cultures of Bacillus sublilis when incubated with DZC. When high levels of reduced glutathione were added to the

cultures the DNA inhibition caused by DZC was reversed. This would be expected, since the diazo compound couples very easily with sulfhydryl groups and also with reactive sites on nucleic acids. Because of this it has been suggested that this action may have something to do with the pharmacological action of DTIC. Therefore, the addition of nontoxic sulfhydryl compounds such as glutathione and cysteine would bind to the DZC and reverse the inhibition of cell growth caused by incubating the cells with DTIC in the presence of light.

Though this metabolite has a strong affinity for nucleophilic groups there is some question as to its ability to be very reactive since it is a dipolar molecule. This could hinder its capability to be transported through the cell membrane and enter the cell. But Loo et al. (31) postulated that if DZC was produced rapidly enough some of it could find a way into the cells which would enable it to interact with the intracellular molecules such as DNA and RNA. Saunders et al. (43) showed in experiments with DTIC-2-C that the diazo compound does indeed enter the cells.

Though DZC has been postulated to be the pharmacologically active form of DTIC (20)(44)(50), Mizuno et al. (37) found contradictory results. Radioactive DTIC was allowed to photodecompose to DZC in the presence of RNA and DNA. These macromolecules showed virtually no radioactivity.

Though the diazo compounds are known to readily couple with many reactive groups it appeared unlikely that the alkylation of nucleic acids occured during this study with S180 tissue. To further support this hypothesis, Mizuno et at. (37) found that in the presence of light, DTIC gave a negative response to a test used for the detection of alkylating agents.

There is another means of activating DTIC via microsomal metabolism *in vivo*. 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MIC) and formaldehyde are formed from DTIC by oxidative N-demethylation (49). In this step the DTIC loses an N-methyl group ultimately as CO_2. MIC then tautomerizes to 5-(3 methyl-2-triazeno)imidazole-4-carboxamide (iso-MIC). This compound spontaneously cleaves to generate 4(5)-diazomidazole-5(4)-carboxamide (AIC), diazomethane (a methylating intermediate) and a methyl carbonium or diazonium ion (50). It is hypothesized that the diazomethane ion and/or the carbonium ion are the agents responsible for the observed methylation of nucleic acids and proteins (31)(36)(37).

Even though light is absent in this reaction pathway, some DZC is generated from DTIC at sufficiently reduced rates. This permits the reactions of the microsomal pathway to take place (17)(31).

MIC is thought to be involved in the methylation of nucleic acids. Mizuno et al. (37) found that DTIC

selectively inhibited DNA synthesis in Sarcoma 180 slices. When the metabolite MIC was tested with the reagent 4-(4-nitrobenzyl) pyridine, it produced a strong positive reaction for the detection of alkylating substances.

In a study conducted by Skibba et al. (51) DTIC, or an active metabolite (MIC), selectively inhibited the incorporation of labeled thymidine into DNA. It did not effect the incorporation of precursors into RNA or proteins. MIC also prevented DNA synthesis in the thymus, liver, spleen and small intestine in normal proliferating rat tissues. However, the study of DTIC on nucleic acid synthesis has shown variable results. In a study by Shirakawa, (47) DTIC was reported to affect RNA and protein biosynthesis more than DNA biosynthesis in L1210 cells. Contradictory results were also reported by Pittilo (41). In this study an analog of DTIC, (methyl 5 (or 4)-(3,3-dimethyl-1-triazeno)imidazole-4 (or 5)-carboxylate was reported to inhibit the growth of gram-positive, gram-negative bacteria, yeasts, filamentous fungi and algae *in vitro.* A concentration of 1.0 µg/ml was sufficient to markedly inhibit the RNA and protein synthesis processes in *E. coli*. A stronger concentration of 5.0 µg/ml totally inhibited these same processes. However, DNA synthesis was unaffected. Cysteine completely reversed the inhibition of this compound for *E. coli* suggesting that a competitive reversal mechanism was involved.

Further studies by Mizuno et al. (36) found that concentrations of MIC greater than 1 x 10^{-3} M inhibited the growth of mouse fibroblast tissue culture cells. In this same study, MIC prevented RNA from incorporating ^{3}H-uridine, and prevented DNA from incorporating ^{3}H-thymidine. There was some evidence that MIC was involved in de novo purine synthesis. When L cells were incubated with ^{3}H-MIC, three purine bases incorporated the radioactive molecule. They were adenine, guanine and 7-methyl guanine. To determine if this effect, caused by MIC on nucleic acid synthesis, was a result of impaired template activity, RNA and DNA polymerase activities were studied. DNA incubated with MIC showed reduced template activity with RNA polymerase but not with DNA polymerase. However, when L cells were incubated with MIC, both DNA and RNA synthesis was inhibited. The conclusion of this study was that MIC-treated cells did impair DNA synthesis but the cause could not be attributed to diminished template activity. It seemed that other factors were involved. It was concluded that the methylation caused the initial error. This then led to additional inactivation which was caused by the production of nonfunctional RNA or proteins.

Other studies support the existence of DNA damage caused by methylation. Strauss et al. (52) reported that methylated DNA caused single strand breaks either directly or by

enzymatic action. Consequently it is possible that the lesions created by the repair of the breaks might be of such magnitude as to prevent the altered DNA from acting as a template for RNA or DNA synthesis. This study also showed that the methylated DNA lost its ability to participate in bacterial transformation.

During another study by Mamet-Bratley (34), it was shown that there could be enough damage caused by methylation to interfere with the biological function of DNA. The altered molecule was shown to reduce the normal template activity for RNA synthesis. There are four postulated theories to explain the effects caused by alkylation on RNA synthesis.

1). The elongation of the chain is slowed down due to steric obstacles that are created by the incorporation of the alkyl groups into the DNA template.

2). The termination of the chain elongation is prematurely stopped.

3). There is an inhibition of the normal enzyme binding process.

4). The initiation of the chain synthesis is prevented.

There is a question as to whether the methylation of RNA or DNA by DTIC can be responsible for the inhibition on DNA synthesis. Roberts et al. (42) showed that mammalian cells were capable of removing alkyl groups from DNA and can carry out a repair synthesis of DNA. For example, the

lesions caused by an alkylating agent such as mustard gas in a DNA molecule could be repaired. The repair mechanism begins by the removal of the alkylated lesions. The cell then replaces the excised portion of the DNA molecule using the remaining undamaged strand of DNA as a template. This repair synthesis is very different from normal semi-conservative replication. There are nucleolylic enzymes that recognize alkylation damage and these enzymes are different from enzymes involved in recognizing UV damage (52). This mechanism, which repairs lesions in the DNA, could account for the resistance of some cells to the cytotoxic effects of alkylating agents.

The microsomal metabolism of DTIC has been shown to cause other effects. In a study by Larson et al. (29) DTIC was able to completely inhibit the breakdown of the low K_m form of c-AMP phosphodiesterase in the supernatant of rat livers. It is well known that high concentrations of intracellular c-AMP can inhibit cell growth. This might be a significant contribution to the pharmacological control of tumor-cell growth since DTIC alters c-AMP levels.

Culver et al. (13) found that DTIC inhibited neuroblastoma cell division. It also increased the activity of three enzymes that are involved in neurotransmitter metabolism. They were tyrosine hydroxylase, choline acelyltransferase and acetylcholinesterase. The cells became

enlarged due to the elevated protein content. DTIC did not affect the intracellular c-AMP levels or the c-AMP phosphodiesterase activity.

Gerulath et al. (16) observed a broad shoulder threshold-type survival curve when chinese hamster ovary cells were incubated with DTIC. This survival curve existed in both light and dark conditions. This suggested that sublethal damage occurs via both metabolic pathways. There is also the possibility that a threshold dose exist below which there seems to be no cell death. This effect could also be explained by the existence of a repair process by which some of the cells are capable of recovering from the sublethal damage. The shoulder might also be caused by pharmacological mechanisms such as membrane exclusion of the drug or metabolic activation of the drug.

Another interesting characteristic of DTIC is its ability to mediate immunogenic changes of experimental tumors. In a study by Nicolin et al. (38) 5178 leukemia cells became more immunogenic when treated with DTIC. When these treated leukemia cells were injected into otherwise susceptible syngeneic hosts, their survival rate was prolonged, sometimes indefinitely. It was suspected that the DTIC treatments induced the appearence of additional new target antigens on the tumor cells rather than increase pre-existent antigenicity in the tumor cells. Similar antigenic changes

were observed in two other leukemia cell lines, L1210 Cr and L1210 Ha by Bonmassar et al. (6). However, the mechanism responsible for the new antigen's appearance is not yet clear. Several mechanisms for its appearance have been hypothesized.

1). The DTIC activates a latent virus which causes the appearance of new virus-coded transplantation membrane antigen(s) (14).

2). The DTIC caused somatic mutations which affect membrane antigens due to its alkylating properties or its ability to bind to nucleic acids. It was postulated by Giampietri et al. (18) that these mutations were caused by one of the above mentioned properties of DTIC. This resulted in the overgrowth of highly immunogenic clones. This then led to the appearance of sublines that carried strong transplantation antigens that could be recognized by hosts that were histocompatible with the parental cell line. These findings are significant since cancer immunotherapy is based on the difference between normal cell antigens and tumor cell antigens.

In summary, there are at least three theories that could explain the mechanism of action of DTIC (9)(13).

1). DTIC has been shown to prevent the synthesis of DNA. This could be due to the sterio-chemical properties of the

drug which somehow causes it to act as a purine analog and thus causes it to interfere with de novo purine synthesis.

2). The metabolism of this drug ultimatly creates a carbonium or diazomethane radical ion which is thought to possess alkylating activity.

3). DTIC or its metabolites are highly reactive to sulfhydryl (S-H) groups. It was shown in many studies that the addition of compounds that contain numerous S-H groups caused competitive reversal of DNA synthesis inhibition or cell growth inhibition.

II. Materials and Methods

CHEMICALS

DTIC (NSC-45388) was obtained from Dome Laboratories, West Haven, Conn. It was dissolved in a 1% citric acid and 0.5% mannitol solution. The initial concentration was 10 mg/ml of DTIC to solution. This solution was sterilized by filtration with a Millipore membrane of 0.22 µm. It was kept on ice and protected from the light. The final concentration used per dish was 1.0 mg/ml.

BCNU (NSC-409962) was obtained from The National Cancer Institute. It was dissolved at 1 mg/ml in distilled deionized water and sterilized by the same method as the DTIC. This solution was also kept on ice and protected from the light. The final concentration used per dish was 1.0 µg/ml.

A stock solution of 1% citric acid and 0.5% mannitol was made and sterilized by the same method as DTIC. This was used as one of the controls.

CULTURES

The mouse melanoma cells have been cultured in the laboratory since 1974. They were originally obtained from a B16 tumor that was growing in a C57BL/6 female mouse. These B16C14 melanoma cells were grown on Dulbeccos Modified Eagle Medium (GIBCO) with 5.0% fetal bovine calf serum

(GIBCO) and 0.1% penicillin streptomycin (GIBCO). The medium (DME5) was sterilized by filtration using a Millipore membrane of 0.22 um. The cells were incubated on P60 dishes (60 mm. diameter) at 37°C and in humidified conditions (5% CO_2 and 100% humidity).

PROCEDURES

The cultures were allowed to grow on P100 dishes (100 mm. diameter) until confluent. The medium was removed and trypsin with EDTA (1x) (GIBCO) was used to detach the cells growing on the bottom of the dish. The cell concentration of the resulting suspension was estimated using a haemocytometer. Two concentrations of cells were made by suspending the cells in the DME5 medium. The final concentrations of cells per dish were 200 cells per dish and 500 cells per dish. The cells were inoculated on to P35 (35 mm. diameter) dishes. Two replicates of each dish were made. Each dish contained a final volume of 2 ml of the seeded medium. The dishes were incubated in the dark for approximately one hour to allow the cells to settle. During this time the drug(s) solutions were prepared, sterilized, kept on ice and protected from the light. The dishes were then removed from the incubator and the drug(s) were added in a dark laminar vertical air flow hood.

The dose schedule appears in TABLE I.

The dishes were incubated in the dark to prevent

photoactivation. On day 2, 3, or 4 according to the schedule, the dishes were removed from the incubator and the medium was removed. Each dish was rinsed two times with 1% calcium-magnesium free phosphate buffer saline (CMF-PBS). This solution was prepared from a stock solution and was sterilized using a Sybron/Nalge type S filter unit.

Fresh medium was added and the dishes were allowed to incubate in the dark for a total of 15 days. At the end of the 15 days the dishes were rinsed with a saline solution (0.9g/100ml distilled deionized water). The cells were fixed and stained with a crystal violet solution containing 10% formalin. The dishes were scanned macroscopically for colonies that contained 50 or more cells. These colonies were marked and counted.

CALCULATIONS

The plating efficiencies for Group I, II, and III were calculated by dividing the average of the number of surviving colonies from the two replicated dishes, divided by the number of original seeded cells per dish. In all calculations, only the 200 cells per dish results were used because the 500 cells per dish tended to be confluent and hence impossible to score. For example, when no drug was added and the medium was changed on day 2, the number of colonies that were found on each of the two dishes were 65 and 66. (Refer to TABLE II). The average of this is 65.5.

This number was divided by 200 (original number of cells per dish) to yield a plating efficiency of 0.3275. (Refer to TABLE III and IV). The plating efficiencies for the control group I were averaged. This average is used in later calculations to derive the observed surviving fraction of cells when the drug(s) are added individually (Group II).

The observed surviving fraction of cells for group II was calculated by dividing each plating efficiency from group II for day 2, 3, or 4 of medium exchange, by the plating efficiency average of the control group I (Refer to Table V). The observed surviving fraction of cells from Group III was also obtained in like manner. For example, when DTIC and BCNU were added on day 0 and DME5 was changed on day 2, the plating efficiency was 0.3375. This number was divided by 0.342 (control average) to yield 0.987 (Refer to Table VI).

The expected surviving fraction of cells from Group III was obtained by multiplying the observed surviving fraction of DTIC treated cells by the corresponding expected surviving fraction of BCNU treated cells from Group III. For example, when DTIC was added on day 0 and the medium was changed on day 2, the surviving fraction, 0.811, was multiplied by the surviving fraction, 0.914, which was obtained when BCNU was added on day 0 and the medium was changed on day 2. This calculation yielded the expected surviving fraction, 0.714, DTIC and BCNU added on day 0 and the medium changed on day 2

(Refer to Table VI).

The percent increase of decrease of the observed surviving fraction of cells from the expected surviving fraction of cells was calculated by dividing the former number by the latter number. This result was subtracted from one and multiplied by one hundred to yield the final number as it appears in the last column of Table VI. This was done for each set of drugs in Group III. For example, when DTIC and BCNU were added on day 0, and the medium was changed on day 2, the observed surviving fraction 0.987, was divided by the expected surviving fraction, 0.741, to yield a 33% increase in the observed surviving fraction from the expected surviving fraction. (Refer to Table VI).

III. RESULTS AND DISCUSSION

Two control groups were established during this experiment, dishes with no drugs and dishes with only the citric acid and mannitol solution. This latter control was used to ensure that killing was caused by the DTIC and not the solution in which it was dissolved. The first control group, in which no drug or solution was added to the seeded medium, produced a plating efficiency average of 0.3350. The second control group, in which only the citric acid and mannitol solution was added produced a plating efficiency average of 0.3480. There is no significant difference in these two averages, indicating that the citric acid and mannitol solution did not cause cell death. The average of these two plating efficiencies was 0.342.

As expected, the DTIC when added alone increased cell mortality. This was shown by the continuous decrease in observed surviving fraction of cells, which started at 0.811 and dropped to 0.139. The longer the drug was left in contact with the cell population, the greater the percentage of cells killed. This is in agreement with Wilkoff's et al. (54) first order kinetics model that was demonstrated for DTIC treated L1210 cells. The rate of viable cell reduction was not dependent on the number of living cells in the population for a given drug concentration and given dose

period.

The results also indicated that this particular cell line, B16CL4, still retained a means to enzymatically activate the DTIC *in vitro*. Thus Miller's et al. (35) hypothesis concerning the lack of enzymes necessary to activate the DTIC in older mammalian cell cultures was not valid for this culture line of mouse melanoma cells. Thus, a S9-mixture of microsomal enzymes from a liver homogenate of mice treated with Aroclor 1254 as described by Ames et al. (1) was not needed.

Many studies have shown that DTIC and/or its metabolites, DZC or MIC, inhibit the growth of bacteria or mammalian cell cultures. (36)(37)(41)(44)(51)(55) It is assumed in this study that the enzymatic metabolic pathway was the one responsible for the activation of the drug(s). This was concluded because the drugs and treated cultures were protected from photodecomposition.

The mechanism of action of this drug has not yet been elucidated. Some hypotheses suggest that DTIC inhibits DNA synthesis (36)(37)(51), RNA synthesis (35)(36)(41)(47), or protein biosynthesis (41)(47). The metabolically active metabolite, MIC, has been shown to impair template activity (52), either by interfering with the RNA polymerase enzymes (36) or the DNA polymerase enzymes. The template activity has also been shown to be affected by the methylation of DNA

single strands, which cause breaks. Although the breaks are repaired, the numerous lesions that are created could interfere with the template activity of DNA.

The plating efficiencies obtained from the experimental addition of BCNU added alone did not significantly differ from one another. Differences observed are probably due to experimental variation.

The purpose of this study was to establish a synergistic dose schedule for DTIC and BCNU. It has been shown by Hill et al. (21) that an enhancement dose schedule does exist when DTIC is administered to female BALB/C x DBA/2 F1 mice bearing Harding-Passey mouse melanoma cells on day 0 and 1-(2-chloroethyl)-3-(4-methylcyclo-hexyl)-1-nitrosourea (MeCCNU) is administered on day 1. When this dose schedule was administered, 90% of the mice were cured. The enhancement of the survival of the mice was not as great when the order of the drugs was reversed. MeCCNU, like BCNU, produces an alkylating moiety and a carbamoylating moiety. These two nitrosoureas posssess similar structures and could have similar mechanisms of action. In another study by Hill et al. (unpublished) enhancement was also observed when DTIC was administered to mice, bearing B16 mouse melanoma cells, on day 0 followed by the administration of BCNU on day 2.

In the present study, the results obtained when DTIC and BCNU were added on day 0, showed a 30% increase of the

observed surviving fraction of cells from the expected surviving fraction of cells. This dose schedule did not produce any killing enhancement. When DTIC was added on day 0 and BCNU was added on day 1, there was a 43% increase of observed surviving fraction of cells from the expected surviving fraction of cells. This dose schedule also did not produce an enhancement of cell death. Significant results were obtained however, when DTIC was added on day 0 and BCNU was added 2 days later. This dose schedule produced a 60% decrease in the observed surviving fraction from the expected surviving fraction. Cell death was enhanced when the drugs were added in this manner. This corresponds closely with the results obtained from the two previously mentioned reports by Hill et al. (21) (unpublished).

These enhanced effects could be caused by the metabolite MIC, which is thought to cause the initial damage to the DNA molecule. The alkylation mechanism could have caused strand breaks in the molecule, thus interfering with DNA or RNA template activity. BCNU then produced a carbamoylation reaction which caused the inhibition of the repair enzymes. A similar effect was observed by Kann et al. (23). In this study CIS interfered with the repair of DNA, which was damaged by an alkylating mechanism caused by x-rays. Fornace et al. (15) found that CIS prevented the rejoining of strand breaks by interfering with the ligase

step of DNA repair. These breaks were caused by exposing the cells to x-rays.

In another study by Shirakawa et al. (47) DTIC produced an effect on the G_2 period in the mitotic cycle. Because of its alkylating abilities, DTIC caused the G_2 phase to be prolonged. This prolongation was responsible for the adverse affect on RNA and protein biosynthesis more than on DNA synthesis. Thus, the content of DNA increased in each cell because of the delay during the G_2 phase. It is during this time that the DNA strands have doubled, but division has not yet taken place. During the same study BCNU was show to produce a prolongation of the S phase of the mitotic cycle. This interfered with DNA synthesis. There was a strong correlation between the delayed S phase and cell death. Both of these drugs have been shown to interfere with different stages of the mitotic cell cyle. Enhanced cellular destruction could have resulted because each of the drugs causes its effects at different times during the growth of proliferating cells.

The results obtained from the BCNU added first trial were expected. When BCNU and DTIC were added on day 0, (in the BCNU added-first trial) the same result, a 33% increase in the surviving fraction, occurred as in the DTIC added-first trial. This was expected since both drugs were added at the same time in both trials. This increase could be

explained by several speculations. Either the drugs were interfering with each other or there was experimental variation in the controls. There was a significant decrease, 66%, in the observed surviving fraction from the expected surviving fraction when DTIC was added 1 day after BCNU. But when DTIC was added 2 days after BCNU, the observed surviving fraction rose to 26% of the expected surviving fraction. It is possible that the DTIC may not have been activated during this dose schedule. There might have been more enhancement if a S9-microsomal mixture had ben included in the experiments. Also, the drug mechanisms may have interfered with each other to cause the observed effects.

APPENDIX 1.

TABLE I.

DOSE SCHEDULE

DAY OF ADDITION OF DRUG(S) OR SOLUTIONS

DTIC	Citric acid mannitol solution	BCNU	DME5
-	-	-	2
0	-	0	2
0	-	-	2
-	0	0	2
-	0	-	2
0	-	1	3
0	-	-	3
1	-	0	3
-	1	0	3
-	0	-	3
-	1	-	3
-	-	-	4
0	-	2	4
0	-	-	4
2	-	0	4
-	2	0	4
-	0	-	4
-	2	-	4

(-) nothing added

Table II.

INITIAL RESULTS

DAY OF ADDITION OF DRUG(S) OR SOLUTIONS				NUMBER OF COLONIES PER DISH					
DTIC	CIRTIC ACID/ MANNITOL	BCNU	DME5	200 CELLS /DISH		AVG	500 CELLS /DISH		AVG
-	-	-	2	65	66	65.5	C	C	-
0	-	0	2	75	60	67.5	91	104	97.5
0	-	-	2	57	54	55.5	99	102	100
-	0	0	2	63	62	62.5	72	C	-
-	0	-	2	69	67	68.0	C	C	-
-	-	-	3	C	C	-	C	C	-
0	-	1	3	19	19	19.0	57	67	62.0
0	-	-	3	21	8	14.5	34	49	41.5
1	-	0	3	21	23	22.0	52	54	53.0
-	1	0	3	79	72	75.5	C	C	-
-	0	-	3	74	74	74.0	C	C	-
-	1	-	3	80	71	75.5	C	C	-
-	-	-	4	65	72	68.5	C	C	-
0	-	2	4	1	6	3.5	3	12	7.5
0	-	-	4	9	10	9.5	14	20	17.0
1	-	0	4	37	37	37.0	96	99	97.5
-	2	0	4	61	62	61.5	C	C	-
-	0	-	4	67		67.0	C	C	-
-	2	-	4	67	65	66.0	C	C	-

(-) nothing added
(C) confluent dish

TABLE III.

PLATING EFFICIENCIES AND
PLATING EFFICIENCY AVERAGES
OF CONTROLS

CONTROLS GROUP I

DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF FRESH MEDIUM	PLATING EFFICIENCY	PLATING EFFICIENCY AVERAGE
none	-	2	0.3275	-
none	-	3	C	-
none	-	4	0.3425	0.3350
citric	0	2	0.3400	-
acid/	0	3	0.3700	-
mannitol	0	4	0.3350	0.3480

average of both control groups is 0.3420

(C) confluent dish

TABLE IV.

PLATING EFFICIENCIES OF DRUG TREATED CELLS

DRUGS ADDED ALONE GROUP II

DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF FRESH MEDIUM	PLATING EFFICIENCY
DTIC	0	2	0.2775
DTIC	0	3	0.0725
DTIC	0	4	0.0475
BCNU	0	2	0.3125
BCNU	0	3	0.3775
BCNU	0	4	0.3075

DRUGS ADDED TOGETHER GROUP III

DTIC ADDED FIRST

DRUG	DAY OF ADDITION OF DRUG	DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF DME5	PLATING EFFICIENCY
DTIC	0	BCNU	0	2	0.3375
DTIC	0	BCNU	1	3	0.0950
DTIC	0	BCNU	2	4	0.0175

BCNU ADDED FIRST

DRUG	DAY OF ADDITION OF DRUG	DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF DME5	PLATING EFFICIENCY
BCNU	0	DTIC	0	2	0.3375
BCNU	0	DTIC	1	3	0.1100
BCNU	0	DTIC	2	4	0.1850

TABLE V.

OBSERVED SURVIVING FRACTION
OF DRUG(S) ADDED ALONE

DRUGS ADDED ALONE GROUP II

DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF DRUG	OBSERVED SURVIVING FRACTION
DTIC	0	2	0.811
DTIC	0	3	0.212
DTIC	0	4	0.139
BCNU	0	2	0.914
BCNU	0	3	1.104
BCNU	0	4	0.899

TABLE VI.

OBSERVED AND EXPECTED SURVIVING FRACTION OF CELLS AND PRECENT INCREASE OR DECREASE OF SURVIVING FRACTION OF CELLS FOR DRUGS ADDED TOGETHER

DRUGS ADDED TOGETHER GROUP III

DTIC ADDED ON DAY 0

DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF FRESH MEDIUM	OBSERVED SURVIVING FRACTION	EXPECTED SURVIVING FRACTION	PERCENT INCREASE/ DECREASE SURVIVING FRACTION
BCNU	0	2	0.987	0.741	33% >
BCNU	1	3	0.278	0.194	43% >
BCNU	2	4	0.051	0.127	60% <

BCNU ADDED ON DAY 0

DRUG	DAY OF ADDITION OF DRUG	DAY OF ADDITION OF FRESH MEDIUM	OBSERVED SURVIVING FRACTION	EXPECTED SURVIVING FRACTION	PERCENT INCREASE/ DECREASE SURVIVING FRACTION
DTIC	0	2	0.987	0.741	33% >
DTIC	1	3	0.322	0.895	66% <
DTIC	2	4	0.541	0.729	26% <

(>) Increase in observed surviving fraction from expected
(<) Decrease in observed surviving fraction from expected

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