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Diane Marie Barnes
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THE CYTOTOXIC AND DNA DAMAGING EFFECTS OF

ETHYLCHOLINE MUSTARD AZIRIDINIUM ION (AF64A)

by

Diane Marie Barnes

A Thesis Submitted to the Faculty of the Graduate School

of Loyola University of Chicago in Partial Fullfillment

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DEDICATION

This thesis is dedicated to my husband, Scott Glait, for his constant encouragement and for always beliving in me.

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CHAPTER I

INTRODUCTION

The purpose of this research was to study the mechanism of action of ethylcholine mustard aziridinium ion (AF64A), a potential cholinotoxin, using an _in vitro_ system. A number of questions can be raised concerning the mechanism of action of AF64A: 1) is its action specific for cholinergic systems? 2) does this drug act selectively on cholinergic neurons? 3) what is the exact mechanism of cell death caused by the drug? 4) how, and why, does it cause, in experimental animals, some of the long lasting behavioral and neurochemical cholinergic deficits observed also in brains of patients with Alzheimer's dementia?

The structure of AF64A makes it a unique compound to use in the study of cholinergic toxicity. Its structural similarity to choline (Ch) increases the probability that the drug will be taken up by the high affinity Ch transport (HAChT) system, and concentrated in cells that are cholinergic in nature. In addition, its aziridinium moiety should render it a potent alkylating agent. Alkylation of DNA can be potentially very toxic to the cell, resulting in decreased mRNA transcription, abnormal DNA replication, or even cell death.

AF64A has been be studied in three different cell lines: a) L1210 cells, obtained from a rapidly dividing mouse leukemia cell line; b) A1235 cells, derived from a non-cholinergic human neuroglioma cell line; and c) LAN2 cells, obtained from a human neuroblastoma cell line known

to possess high levels of the enzyme Ch acetyltransferase (ChAT). ChAT is the enzyme responsible for the catalytic conversion of Ch to acetylcholine (ACh). Colony formation assays have been performed to determine whether AF64A produces a differential cell kill among these three cell lines. DNA damage caused by this agent has been assayed by the DNA alkaline elution technique. The amount of DNA single strand breaks caused by this agent has been quantified. Levels of ChAT, thought to be a target of AF64A, have been determined prior to and following exposure to AF64A.

Using cells in tissue culture, it should be possible to determine if AF64A is more toxic to a cell line which is cholinergic than to non-cholinergic cells. In addition it may be possible to further study the intracellular mechanisms of AF64A induced cholinotoxicity.

CHAPTER II

REVIEW OF THE RELATED LITERATURE

A. ALZHEIMER'S DISEASE

Alzheimer's disease is a neurodegenerative disorder characterized by behavioral, anatomical and neurochemical changes in the brain. In a recent review article, behavioral changes in Alzheimer's disease have been described as "an intellectual deterioration occurring in an adult, that is severe enough to interfere with occupational or social performance...The cognitive changes include not only disturbances in memory but also disturbances in other cognitive areas, such as language use, perception, ability to learn necessary skills, solve problems, think abstractly and make judgments (Katzman, 1986)." Approximately 60 different disorders are associated with dementia, but Alzheimer's type dementia accounts for about 50-60 percent of all cases of dementia (Katzman, 1986).

1. Anatomy

Anatomically the major brain areas found to be affected in Alzheimer's disease are the frontal and temporal cortices, entorhinal cortex, amygdala, locus coeruleus, and cholinergic neurons of the nucleus basalis of Meynert, medial septum and diagonal band of Broca (Schneck et al., 982; Katzman, 1986). Cell loss and degeneration with loss of cholinergic markers such as acetylcholine (ACh), choline

acetyltransferase (ChAT) and acetylcholinesterase (AChE), as well as the histopathological changes characteristic of the disease, occur in these areas (Bowen et al., 1976; Davies, 1978; Coyle et al., 1983; Hyman et al., 1984; Perry et al., 1984; Ichimya et al., 1986; Weber, 1986).

2. Neurochemistry

   A number of neurotransmitter systems have been shown to be altered in Alzheimer's disease including a decrease in ChAT and glutamic acid decarboxylase (GAD) activities in human cortex (Bowen et al., 1976; Davis et al., 1985); a decrease in ChAT activity in several brain regions (Davies, 1978); as well as decreases in noradrenaline in the locus coeruleus (Ichimiya et al., 1986). Neuropeptide Y and somatostatin have also been found to be reduced due to a reduction of fiber plexuses (Kowall and Beal, 1988). Of these the most consistent observation has been a significant decrease in cholinergic function (reviewed in: Bartus et al., 1982; Coyle et al., 1983; and Katzman, 1986). This has led to the proposal of a cholinergic hypothesis of Alzheimer's disease, which states that the deficits seen in Alzheimer's disease are due to a degeneration of cholinergic regions of the brain and a decrease in cholinergic markers such as ACh, ChAT and AChE (Bartus et al., 1982). The decrease in ACh levels was partially due to a decrease in the number of cells containing ACh, particularly in the nucleus basalis of Meynert (Coyle et al., 1983).

   Additionally, changes have been found in the ChAT enzyme, the major enzyme involved in the synthesis of ACh (Koshimura et al., 1986). Not only was there a decrease in the amount of ChAT present in the

brain, but there also were changes in the efficacy of the enzyme (Koshimura et al., 1986). V_{max} for ChAT was found by Koshimura et al. (1986) to be decreased in all cortical areas examined in Alzheimer's brains, when compared to age-matched controls. The affinity of the enzyme for Ch and acetyl coenzyme A (AcCoA) was significantly decreased in both the frontal and temporal cortex. The isozyme pattern, which measures the activity of different fractions of ChAT separated by column chromatography on a Sephadex G-200, was also different in the Alzheimer's brains. This author postulated an alteration in the structure of the ChAT protein in Alzheimer's patients.

3. Pathology

Recent advances in immunohistochemistry and molecular biology have provided evidence for a genetic component to Alzheimer's disease. The cerebrovascular amyloid protein from the brain of an adult has been isolated and purified (Glenner et al., 1984). Amino acid sequence analysis has shown the protein to be homologous to the beta amyloid protein isolated from cerebrovascular amyloid fibril protein in Alzheimer's patients (Glenner et al., 1984). Amyloid fibers have also been found in the cores of senile plaques and intracellularly in the paired helical filaments (PHF), both of which are characteristic of the pathology of Alzheimer's disease (Selkoe et al., 1986). The cDNA encoding for the beta amyloid protein has recently been cloned, sequenced and mapped to chromosome 21 (Goldgaber et al., 1987; Tanzi et al., 1987). Delabar et al. (1987) reported that a duplication of a subsection of chromosome 21 may the critical genetic defect in

Alzheimer's disease, although other investigators were unable to find duplication of chromosome 21 in Alzheimer's disease (St. George-Hyslop et al., 1987).

The other protein found to be associated with pathological changes in Alzheimer's disease is the Tau protein which is found in the PHF and neurofibrillary tangles in Alzheimer's brains (Kosek et al., 1986) and neurofibrillary tangles and neuritic plaques (Wood et al., 1986). A study by Grundke-Iqbal et al. (1986) suggested that the Tau protein in Alzheimer's patients may be abnormally phosphorylated because antibodies to Tau did not react with the protein from Alzheimer's brains until the protein had been dephosphorylated, although the antibody reacted with Tau protein from controls which had not been dephosphorylated. Recently the cDNA clones for the Tau protein have been isolated and mapped to chromosome 17 with an additional region of homology on chromosome 6 (Neve et al., 1986).

Lymphocytes from Alzheimer's patients have been shown to be deficient in certain types of DNA repair. Lymphocytes from Alzheimers's patients were able to repair damage induced by u.v. light, but were unable within 3 hours to repair damage induced by the alkylating agent methyl methane sulfonate (MMS) (Robison et al., 1987), although others have found repair to be equal to normal cells at 24 hours (Kinsella et al., 1987). Decreased levels of mRNA and protein synthesis have also been associated with Alzheimer's disease (Sajdel-Sulkowska et al., 1984).

Thus behavioral, neurochemical and neuropathological changes are seen in Alzheimer's brains. These may or may not be associated with a

genetic defect and the precise defect is presently unknown. Increased

levels of <u>beta</u> amyloid protein or <u>Tau</u> protein may either be a

consequence of cell death or may result in cell death. Abnormal

phosphorylation of these proteins may lead to cell death. Defects in

DNA repair mechanisms may lead to decreases in mRNA transcription and

ultimately to decreased protein synthesis, including enzymes such as

ChAT, in Alzheimer's patients. What is known is that these changes

occur and that there may be a predisposing genetic defect which might

lead to neuronal cell death in certain subpopulations.

B. ETHYLCHOLINE MUSTARD AZIRIDINIUM ION (AF64A)

The synthesis of ACh is dependent on two processes: 1) the uptake

of Ch through a high affinity uptake mechanism (HAChT), the rate

limiting step in the synthesis of ACh; and 2) the presence of ChAT, the

enzyme which acetylates Ch using AcCoA as a substrate. AF64A (Fig.1),

which bears structural similarity to Ch, has been shown to be a potent

cholinotoxic agent through inhibition of HAChT and depletion of ChAT.

1. High affinity choline transport

Two distinct Ch transport mechanisms have been demonstrated using

rat brain synaptosomes. One is a high affinity Ch transport site which

had a $K_m - 1 \times 10^{-6}$, and the other is a low affinity Ch transport site,

which had a $K_m - 9 \times 10^{-5}$ (Yamamura and Snyder, 1972). The high

affinity transport system required sodium and was associated with the
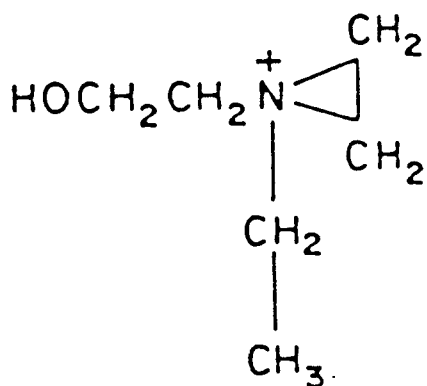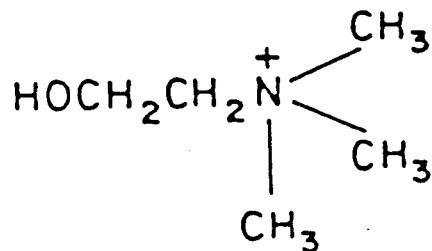
FIGURE 1. Chemical representation of ethylcholine aziridinium (AF64A) and its structural analog choline.

formation of ACh. The other was less sodium dependent and was not associated with ACh synthesis (Yamamura and Snyder, 1972).

In an early study by Rylett and Colhoun (1980), AF64A was shown to inhibit uptake of Ch into rat brain synaptosomes in an irreversible fashion with a $K_i = 2.72$ uM. It was hypothesized that the aziridinium portion of the AF64A molecule might be a potent alkylator of the carrier protein. A later study by Curti and Marchbanks (1984) confirmed that the inhibition of Ch uptake by AF64A was irreversible. However, they found a K_i approximately seven times higher than the values obtained by Rylett and Colhoun, 20 uM, with a concentration dependency which reached a plateau at 100 uM. A recent study has demonstrated that low concentrations of AF64A produced reversible inhibition ($IC_{50} = 1.35$-2.25 uM) and that higher concentrations produced irreversible inhibition of HAChT ($IC_{50} = 25$-30 uM) (Pittel et al., 1987). These authors also demonstrated that HAChT was more sensitive than the low affinity Ch transport to inhibition produced by AF64A. They also postulated that the carrier protein was alkylated in the outward conformation.

2. Choline acetyltransferase

ChAT is the enzyme necessary for the conversion of acetylCoA and Ch to ACh. Human ChAT, as measured by immunoaffinity purification has a molecular weight of approximately 68,000 daltons (Bruce et al., 1985). This figure is in agreement with other authors who generally report a value of 66,000 to 70,000 D (McGeer et al., 1984). In another study. it was shown that ChAT exists in 2 forms: a soluble form derived from the disruption of cholinergic perikarya, axons and dendrites, and a

particulate form which is bound to the membranes of cholinergic nerve terminals. The soluble form accounts for about 60% of ChAT and the membrane bound form accounts for about 40% of ChAT. When separated by SDS-PAGE, the membrane bound ChAT was found to separate into two bands, with molecular weights of 67,000 and 62,000 daltons. Both forms of ChAT had approximately the same specific activity (Peng et al., 1986).

ChAT activity was irreversibly decreased by AF64A in rat hippocampus (Mantione et al., 1983); in rat-mouse neuroblastoma-neuroglioma cells (NG108-15) (Sandberg et al., 1985); chick embryo neuron enriched cultures (Davies et al., 1986); in primary neuronal cultures isolated from rat brain (Atterwill et al, 1986; Pillar et al., 1988); and in various regions of rat brain (Leventer et al., 1987). Sandberg et al. (1985), using partially purified enzymes isolated from NG108-15 cells grown in tissue culture, found that AF64A inhibited a number of enzymes involved in Ch metabolism e.g. ChAT, AChE, Ch kinase and Ch dehydrogenase, but not enzymes involved in other cellular processes such as lactate dehydrogenase, alcohol dehydrogenase, carboxypeptidase A or chymotrypsinogen. However, decreases in ChAT activity were not reported at doses below 100 uM following a one hour exposure in vitro, in primary neuron enriched cultures (Koppenaal et al., 1986); or 50 uM, using purified enzyme (Barlow and Marchbanks, 1984). Barlow and Marchbanks (1984) reported that only Ch dehydrogenase activity decreased when cholinergic enzymes were exposed to AF64A in the micromolar range.

3. Neurophysiology

Neurophysiological studies in cats have demonstrated that AF64A
acts presynaptically at cholinergic nerve terminals (Mantione et al.,
1983a). Nictitating membrane contractions, elicited by preganglionic
stimulation, were diminished in a dose dependent manner after injection
of AF64A into the carotid artery, but were unaffected following
treatment with postganglionic cholinergic agonists (Mantione et al.,
1983a). Postganglionic action potentials from the superior cervical
ganglion evoked by preganglionic stimulation were also abolished by
AF64A. However, action potentials produced by postganglionic electrical
stimulation were unchanged by AF64A treatment (Mantione et al., 1983a).
It was thus concluded, based on these studies, that the toxin acted
presynaptically, at the cholinergic nerve terminal (Mantione et al.,
1983a).

In vivo studies have been performed to determine whether AF64A
could induce the persistent cholinergic deficits associated with
Alzheimer's disease (Mantione et al., 1981, 1983a, 1983b, 1984; Jarrard
et al., 1984; Walsh et al., 1984; Leventer et al., 1987; Hortnagl et
al., 1987; Chrobak et al., 1987). Following intracerebroventricular
(icv) injection of 65 nmoles of AF64A into mice there was a loss of
functional, sodium-ion dependent, HAChT sites in the cortex and
hippocampus (Mantione et al., 1981). More recently, using as little as
3 nmoles AF64A/3 ul/ventricle in rats, Leventer et al. (1987) showed
similar reductions in ACh, ChAT, AChE and HAChT in the hippocampus up to
one year following administration of AF64A (Leventer et al., 1987).

4. Learning and memory deficits

In addition to the neurochemical changes observed, AF64A was able to disrupt memory in rats (Walsh et al., 1984; Chrobak et al., 1987; Tateishi et al., 1987; Mouton et al., 1988; and Nakamura et al., 1988). In the study by Walsh et al. (1984) AF64A selectively depleted ACh in hippocampus and cortex and produced long-term passive avoidance and radial-arm maze deficits in the rat. AF64A was shown to transiently impair retention on a passive avoidance task 35 days after dosing and permanently impair learning of a radial arm maze task 60-80 days after dosing. Losses in cholinergic parameters also occurred in these animals up to 120 days after AF64A administration. Following administration of AF64A, bilaterally (3 nmoles, icv) to rats, the ability of the animal to perform a working memory task, which measured trial specific information, was markedly impaired in the AF64A treated group, which did not recover even when the animal was able to perform a reference memory task which measured trial independent information (Chrobak et al., 1987). Neurochemical parameters measured in these animals at 42 days after dosing showed cholinospecific changes including a decrease in hippocampal ChAT activity. This appeared to be specific to the hippocampus and did not show any decrease in ChAT activity in the frontal or parietal cortex, striatum, cingulate gyrus or amygdala. Levels of catecholamines and indoleamines and their metabolites also were unchanged (Chrobak et al., 1987). Tateishi et al. (1987) demonstrated a decrease in presynaptic cholinergic markers and decreased latencies on a step down passive avoidance task following intrahippocampal injections. Mouton and coworkers (1988) found marked

memory retention deficits on a passive avoidance foot shock test 24 and 48 hours after intracerebral injection (1 nmol/1 ul) to the frontal-parietal cortex. Cholinergic parameters were also found to decreased. The authors concluded that the damage generated was specific to the cholinergic system while larger injections caused non-specific damage. Injections to the basal forebrain of 1 nmol AF64A impaired acquisition and retention of a T maze task which also appeared to be cholinospecific (Nakamura et al., 1988).

5. Specificity

Not all workers agree that AF64A is cholinospecific (Jarrard et al., 1984; McGurk et al., 1987). Although Jarrard et al. (1984) obtained results that were similar to those obtained by Walsh et al. (1984), some non-specific damage to various brain regions had been found (Jarrard et al., 1984). However, subsequent studies (Hortnagl et al., 1987) have shown that the effect of AF64A is dose-specific. Thus, the earlier reports, conducted with high concentrations of the compound may indeed have had some non-specific effects. More recent studies, using considerably lower doses of AF64A (Hortnagl et al., 1987; Chrobak et al., 1987), have demonstrated a cholinoselective action, as well as behavioral deficits, following AF64A administration.

Other studies using systems other than rat brain have also shown that AF64A is cholinospecific. Injections of AF64A into chick retina (Millar et al., 1987) and goldfish retina (Villani et al., 1988) showed specific reductions in cholinergic parameters while leaving other transmitter systems relatively intact. Both authors concluded that

AF64A was cholinospecific. Amir et al. (1988) have recently
demonstrated that AF64A is cholinospecific at doses lower than 22.5 uM
in primary cultures isolated from rat brain, but caused non-specific
damage at higher concentrations. Thus, it can be concluded that AF64A
has selective cholinotoxic actions at lower concentrations and generates
non-specific damage at higher concentrations (Amir et al., 1988).

Thus administration of AF64A to rats has been shown to cause
behavioral and neurochemical changes, which are comparable to some of
the changes seen in patients afflicted with Alzheimer's disease. The
proposed mechanism of action of AF64A is a persistent, cholinergic
hypofunction induced primarily by inhibition of HAChT, and an eventual
loss of ChAT (Mantione et al., 1984). However this hypothesis does not
account for the long term changes following administration of AF64A,
including permanent depletion of ChAT and cell death.

C. DNA DAMAGING AGENTS AND REPAIR

  1. DNA damage by aziridinium compounds

     The structure of AF64A contains an aziridine ring which has been
proposed to be the portion of the molecule responsible for its
cholinotoxicity (Hortnagl et al., 1988). The aziridine ring is known to
be a powerful DNA alkylating moiety and many antitumor agents are
derivatives of aziridine, including 3,6-diaziridinyl-2,5-bis
(carboethoxyamino-1,4-benzoquinone) (AZQ) which was shown to be a
bifunctional alkylating agent with some activity against brain tumors
(Szmigiero et al., 1984). Nitrogen mustard, one of the oldest antitumor
agents, is believed to work through an ethyleneimmonium ion intermediate

to form guanine N^7-monoalkylation products as well as crosslinks between the N^7 position on two guanines in DNA (Boyland, 1946; Hanby et al., 1947; Brookes and Lawley, 1961; Lawley and Brookes, 1963; Kohn and Spears, 1968). Ethyleneimine, structurally very similar to the aziridinium portion of AF64A, has been shown to alkylate the N^7 position in guanosine and deoxyguanosine (Hemminki, 1984). N,N-diethyl-chloroethylamine, which varies from AF64A only in the substitution of the side chain hydroxyl group with a hydrogen atom, was shown in vitro to preferentially alkylate the N^7 position on guanine (Price et al., 1968). Aziridineethanol, again structurally similar to AF64A, was shown to preferentially alkylate the N^7 position on guanine and guanosine as did nornitrogen mustard (Hemminki, 1987). Computer generated models of drug interaction with double stranded DNA confirmed that N^7 was the probable site of alkylation by an aziridinium ion (Pearlstein et al., 1980; Kikuchi and Hopfinger, 1980).

There have been several studies which have examined the adducts formed by nitrogen mustard and phosphoramide mustard and the eventual decomposition of these adducts (Mehta et al., 1980; Chetsanga et al., 1982; Kallama and Hemminki, 1984; Muller and Eisenbrand, 1985; Kallama and Hemminki, 1986). Mehta and coworkers (1980) reported that the major adduct formed by reacting phosphoramide mustard with guanosine in aqueous solution at 37° and pH 7.4 was a phosphoramide-mustard-deoxyguanosine, with the mustard bound to N^7 which had a half-life of 2.3 hours. Later, Chetsanga et al. (1982) reported generation of several alkylated guanosine derivatives following reaction with phosphoramide mustard at physiological pH, including three fractions of

phosphoramide mustard-imidazole ring-opened deoxyguanosine complexes, which were referred to as: phosphoramide mustard-formamido-pyrimidine (PM-FAPy), phosphoramide mustard-deoxyguanosine, and phosphoramide mustard dideoxyguanosine as well as intact phosphoramide mustard deoxyguanosine and phosporamide mustard dideoxyguanosine complexes. Alkylation at the N^7 position of guanosine caused labilization of the imidazole ring, resulting in a ring-opened FAPy derivative which could be removed by FAPy-DNA glycosylase leading to an apurinic site (Chetsanga et al., 1982).   In the work done by Price and coinvestigators, the monofuctional mustard also yielded a mustard bound to guanine at the N^7 position and mustard bound to an imidazole ring-opened guanine when deoxyguanosine was treated at 40° and pH 7 (Price et al., 1968).   Mueller and Eisenbrand (1985) found that there was a correlation between the electron withdrawing effects of the alkyl substitutions and the rate of imidazole ring opening and subsequent depurination.

   2. Repair

        Repair of alkylation induced damage is generally thought to occur via enzymatic removal of the ring opened, damaged base by the FAPy glycosylase (Margison and Pegg, 1981; Chetsanga et al., 1982) or 7-methylglysosylase (Margison and Pegg, 1981).   The ultimate result was an apurinic site in the DNA.   An enzyme known as DNA insertase has been shown to have the ability to insert the appropriate purine base into the apurinic site (Deutsch and Linn, 1979).   It has also been shown that other enzymes involved in excision repair can play a role in repair of

alkylation damage via both short and long patch repair (Snyder and Regan, 1982). Also the ring destabilization can lead to depurination by the FAPy glycosylase, leaving an apurinic site where incorrect Watson-Crick base-pairing may lead to incorrect bases being incorporated into the DNA (Springgate and Loeb, 1973; Shearman and Loeb, 1979; Kunkel, 1984). In fact it has been shown that both nitrogen mustard and nornitrogen mustard can be mutagenic (Hartly-Asp and Hyldig-Nielsen, 1984). The apurinic site may also result in DNA single strand breaks because of inhibition of DNA synthesis (Boiteaux and Laval, 1983).

The findings described above could potentially explain the long term cholinergic hypofunction following exposure to AF64A. AF64A is structurally similar, on one hand, to Ch, and on the other hand to agents known to cause DNA damage. If the DNA in cholinergic neurons has been damaged by exposure to an agent which can cause DNA lesions resulting in mutations, the system may not be able to transcribe message to regenerate the inactivated enzymes. This might result in a long term depletion of ChAT and possibly enzymes essential to the cell's survival, and ultimately cell death.

The studies conducted in this thesis research were performed on cells grown in tissue culture, which offered several advantages not available in animal studies. The cell lines studied were selected for the trait of interest, in this case cholinergic vs. non-cholinergic. Secondly, conditions of drug treatment, such as the concentration of drug, duration of exposure, and amount of Ch present in the media were carefully controlled. Lastly the amount of cytotoxicity and DNA damage produced by AF64A was able to be quantified.

MATERIALS AND METHODS

A. DRUG PREPARATION

AF64A was prepared according to Fisher et al. (1982). The reaction occurs as follows:

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-O-CH_2-CH_2-\overset{\overset{\displaystyle Et}{|}}{N}-CH_2-CH_2-Cl \cdot HCL \xrightarrow[\text{2)HCL->pH 7.4}]{\text{1)NaOH,pH 11.5}} HO-CH_2-CH_2-\overset{\overset{\displaystyle Et}{|}}{N^+}\!\!\!<\!\overset{\displaystyle CH_2}{\underset{\displaystyle CH_2}{}}$$
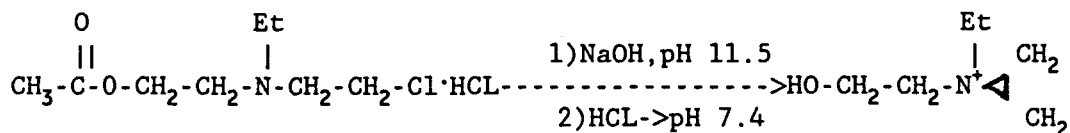
Briefly acetoxy-AF64A·HCl (FW=230) was weighed on a Mettler AE 163 analytical balance. Drug was dissolved in sterile filtered, double distilled H_2O to a final concentration of 0.01M. The pH of the mixture was adjusted to 11.5 with approximately 5 ul/ml 10N NaOH in initial L1210 experiments, or 10 ul/ml 4N NaOH in later experiments which allowed greater control over the pH. This alkalinization step led to hydrolysis of the starting material. The mixture was stirred for 30 minutes on a stir plate at room temperature. The pH was monitored during this time using pH test strips (EM Scientific) and maintained at 11.5 using 4N NaOH. After 30 minutes the reaction was stopped by the addition of concentrated (12N) HCl (approximately 5 ul/ml). The pH was

B. CELL CULTURE

The three cell lines studied were LAN2, a human neuroblastoma cell line which has been reported to be cholinergic (West et al., 1977); A1235, a human neuroglioma cell line; and L1210, a mouse leukemia cell line. Cells were maintained as follows:

1. LAN2 and A1235 cells

LAN2 and A1235 cell stocks were subcultured on a weekly basis. Media was poured off cells and cells were rinsed with a Hank's Balanced Salt Solution (HBSS) (Hazleton) containing 0.02% NaEDTA + penicillin (50 U/ml)-streptomycin (50 ug/ml) (Hazleton). Cells were then trypsinized using 2 ml of an HBSS solution containing 0.05% trypsin (Gibco) and 0.02% NaEDTA. Cells were resuspended in 20 ml of complete Eagle's Minimum Essential Media (MEM) which is composed of MEM (Hazleton) + 10% fetal bovine serum (A1235) (Hazleton) or 15% fetal bovine serum (LAN2) + 1 mM glutamine (Hazleton) + 50 ug/ml gentamycin (Schering) + 0.02 M 4-(2-hydroxyethyl)-1 piperazine ethanesulfonic acid (HEPES) buffer (Hazleton), 1 mM sodium pyruvate (Hazleton), vitamin B12 (1.36 ug/ml) (Sigma), D-biotin (.1 ug/ml) (Sigma), and non-essential amino acids (1X) (Hazleton). Cell counts were performed on a Coulter Counter Model ZBI and cells were plated at a concentration of 10^6 cells per flask (Corning 75 ml^3 #25111-75) and grown in complete MEM. The LAN2 and A1235 cells divided several times during the week to achieve final concentrations of approximately 10 million and 20 million cells respectively.

2. L1210 cells

L1210 - Exponentially growing L1210 cells were subcultured daily during the week. Each day the cells were counted on a Coulter Counter Model ZBI and were diluted to a concentration of 3×10^5 cells/ml in 60 ml of medium [Roswell Park Memorial Institute (RPMI) 1630 (Hazleton) + 1 mM glutamine (Hazleton) + penicillin (50 U/ml)-streptomycin (50 ug/ml) (Gibco) + 15% fetal bovine serum (Hazlton)] in Corning roller bottles (1 liter) in a 37° C incubator on a roller apparatus. The doubling time was approximately every 12 hours and cells divided to a final concentration of 10^6 to 1.2×10^6 cells/ml. On weekends cells were seeded at 2×10^4 cells/ml and reached a density of 1×10^6 three days later.

C. COLONY FORMING ASSAYS

AF64A cytotoxicity to tumor cells grown in tissue culture has been demonstrated previously by a lactate dehydrogenase assay which measures cell lysis (Sandberg et al., 1985). In the present study, colony forming assays, which measure the ability of cells to replicate following exposure to drug, were used. This assay allows quantification of the number of intact cells able to replicate and form colonies.

The three different cell lines were exposed to various concentrations of drug for one or two hours, in order to determine the cytotoxic effects of AF64A on the cell lines. These studies were performed as follows:

1. LAN2 and A1235 cells

LAN2 and A1235 cells were seeded at a density of 150 to 30,000

cells per flask and each density had 3 replicate flasks. Cells were
allowed to attach to the flask for 12-16 hours prior to treatment.
Cells were treated in 25 cm^2 flasks in either complete MEM or HBSS
media, for 1 or 2 hours, depending on the experiment, in a 37° (C)
incubator. Drug concentrations were 25 to 100 uM for LAN2 and 50 to 500
uM for A1235 cultures. Drug was aspirated off the cells with vacuum,
and 7.5 ml of fresh medium added. Cells were grown for 10 days (A1235)
or 3 weeks (LAN2). Colonies were then fixed with absolute methanol and
stained with methylene blue [0.2%]. Colonies were air dried, counted,
and the plating efficiency and surviving fraction determined. Plating
efficiency was determined by the number of colonies surviving in
untreated control cultures, divided by the number originally plated in
the flask. The surviving fraction was determined by the number of
colonies surviving drug treatment, divided by the number of colonies in
untreated, control cultures.

   2. L1210 cells

        Exponentially growing cells were treated at a concentration of
500,000 cells/ml in 5 ml of RPMI medium or HBSS (Hazleton) (pH 7.4).
The cells were treated with AF64A at concentrations ranging from 25 to
100 uM. The cells were then spun at low speed on a cell rotator for one
hour in a 37° incubator. At the end of drug exposure, the cells were
centrifuged at 800 x g for 5 minutes and washed three times. Cells were
counted using a Coulter Counter (model ZBI). Following serial dilution,
concentrations ranging from 10^2 to 10^5 cells/ml, 1 ml of the cells were
added to 3 ml of RPMI + 15% FCS + penicillin-streptomycin and 0.1% soft

agar (Difco-Noble agar), according to the method of Chu and Fisher (1968) and grown in Falcon culture tubes (12 x 75 mm, #2058). Surviving colonies were counted at 8-9 days, and the number of colonies surviving in drug treated cultures were compared to untreated control cultures. In order for a particular dilution series to be considered reliable, colonies had to be present in 2 of three replicate culture tubes. The plating efficiency and surviving fraction were determined.

3. Choline protection studies of AF64A induced cytotoxicity

If AF64A and Ch are both taken up via the same Ch transport system, Ch should compete for uptake into the cell and possibly protect the cell from damage caused by AF64A (Davies et al., 1986). In order to investigate that possibility, LAN2 cells were treated with AF64A in HBSS and varying concentrations of Ch, ranging from 10^{-6} M to 10^{-2} M. L1210 cells were treated in HBSS and Ch at the following concentrations: 2.15 x 10^{-5} M, 2.15 x 10^{-4} M, and 2.15 x 10^{-3} M. The doses of Ch were selected because the concentration of Ch in RPMI 1630 is 2.15 x 10^{-5} M, and it was of interest to determine whether this Ch concentration affected the sensitivity of the cells to AF64A when the cells were treated in RPMI 1630. Colony forming ability and surviving fraction were determined as previously described.

D. ALKALINE ELUTION STUDIES

1. Measurements of DNA single strand breaks

The DNA alkaline elution technique (Kohn et al., 1981) was used to study the production of DNA damage generated by AF64A. The structure of

AF64A suggested that it could attack DNA as a monofuctional alkylating agent (Price et al., 1968). Monofunctional alkylating agents have been shown to generate DNA single strand breaks (SSB) in vitro (Lawley and Brookes, 1963; Price et al., 1968), which can be quantified by DNA alkaline elution (Kohn et al., 1981).

The method of alkaline elution has been described by Kohn, et al. (1981). Each cell line was radioactively labelled with 0.02 mCi/ml ^{14}C thymidine (New England Nuclear, 59.3 mCi/mmol). Initially, label is incorporated into short chain DNA, which elutes at a more rapid rate than long chain DNA, and might appear as SSB. Therefore the cells are labelled and then given a cold chase period in which the labelled DNA is incorporated into long chain DNA, which because of its higher molecular weight, will be retained longer on the filter. Also because of differences in the doubling time, which is proportional to the rate of incorporation of radiolabel, the cells were labelled for different periods of time as follows: 24 hours for L1210; 36 hours label with 36 hour cold chase for A1235; 72 hour label with 72 hour cold chase for LAN2. The L1210 cells were not given a cold chase period, but most of the radiolabel is depleted from the media within the first 12 hours and the second 12 hours fulfills the cold chase requirement. Following exposure to AF64A, attached cell lines (LAN2 and A1235) were harvested by scraping with a rubber policeman and resuspended in MEM + 2% FCS. L1210 cells were harvested at various time intervals and resuspended in ice cold RPMI 1630 + 15% FCS. L1210 cells, radiolabelled with 0.05 uCi/ml ^{3}H thymidine (New England Nuclear, 20 Ci/mmole) and receiving Gy gamma irradiation (Gammacell 1000, Atomic Energy of Canada) at 0° C,

were added to the drug treated cells as an internal standard to monitor abnormalities in filters and pump speeds.

Approximately a total of 500,000 drug treated cells (^{14}C) and internal standard cells (^{3}H) were layered on to a 0.8 um pore size, polycarbonate filter (Nuclepore^{R}) under mild vacuum. The cells were lysed using a solution containing 2% sodium dodecyl sulphate (BDH Chemicals Ltd., Poole, England), 0.1 M glycine and 0.025 M EDTA, pH = 10.0. After lysis, 0.5 mg/ml proteinase K (EM Scientific) dissolved in lysis solution was used to remove any residual protein. A tetrapropylammonium hydroxide (RSA) solution containing 0.02 M EDTA and 0.1% SDS with the pH adjusted to 12.1 was pumped through the filter at a rate of 2 ml/hr for 15 hours. The DNA is eluted from the filter as a function of molecular weight, with low molecular weight DNA eluting early, and high molecular weight DNA being retained on the filter. The amount of radioactivity collected in each of five three hour fractions, as well as any radioactivity remaining in the line or on the filter, was determined by liquid scintillation counting in a Beckman LS 5800 using Beckman Readi-Solv MP and corrected for quenching. After the amount of radioactivity in each fraction had been determined, the amount of ^{14}C retained on the filter was computed and plotted against the amount of ^{3}H retained on the filter. Decreasing amounts of ^{14}C retained indicated increasing numbers of single strand breaks.

Radiation also produced single strand breaks in DNA, and these breaks can be quantified by DNA alkaline elution. One rad gamma irradiation generates approximately 1.1 breaks per 10^{9} nucleotides in DNA (Kohn et al., 1976). Using the DNA alkaline elution technique to

compare drug treated cells to those receiving radiation, it was possible

to quantate AF64A-induced DNA damage, which appears as single strand

breaks (SSB) under alkaline conditions, in terms of rad equivalents.

One rad equivalent is the DNA break frequency generated by 1 rad gamma

irradiation. Therefore, this set of experiments utilized alkaline

elution to quantify the amount of strand breaks caused by the drug in

terms of radiation equivalents according to the following equation

(Szmigiero et al., 1984):

DNA break frequency in RAD equivalents -

$$[\log(r_x/r_0)]/[\log(r_{300}/r_0)] \text{ X } 300R$$

where: r_0 - the amount of control DNA retained on the

filter

r_x - the amount of drug treated DNA retained on

the filter

R_{300} - the amount of DNA receiving 300 rad

irradiation retained on the filter

The R_{300} values of several experiments were averaged separately

for each cell line and used in the equation. In cases where the value

for individual experiments was available, that value was used. There

was little variability of the retention of the DNA receiving 300 R at

50% retention of the internal standard DNA e.g. .503 ± .048 for L1210

cells.

2. Repair of AF64A induced DNA damage

Several types of experiments were performed using this technique.

The initial amount of DNA damage was studied immediately after removal

of AF64A. The capacity of the cell to repair the damage was studied 6, 12, or 24 hours after 1 or 2 hour AF64A exposure, by determination of the persistence of single strand breaks.

3. Assays for alkali-labile sites

Using eluting solutions of different pH, the DNA alkaline elution technique can be utilized to study the nature of the DNA damage. Alkylated DNA bases lose stability under alkaline conditions and may depurinate leaving a base free site, which when exposed to alkali will lead to the formation of breaks (Bayley et al., 1961) These are referred to as alkali-labile sites. Following exposure to alkylating agents, DNA elution profiles may be altered if the elution is performed at a higher pH (Kohn et al., 1981). Therefore, alkaline elution was performed with a tetrapropylammonium hydroxide solution at pH 12.1 or pH 12.6, in order to determine whether AF64A induces formation of alkali-labile sites in the DNA. The elution profiles of LAN2 cells exposed to AF64A were compared to those of methylnitrosourea (MNU), which is known to efficiently produce alkali-labile sites (Erickson et al., 1978), and methyl methanesulfonate (MMS) which is known to produce few alkali-labile sites (Wani and D'Ambrosia, 1986), in order to test the ability of AF64A to produce alkali labile sites.

4. Choline protection of AF64A-induced DNA damage

It has been shown that Ch protects cultured neurons from the toxic effects of AF64A (Davies et al., 1986). Thus, if DNA damage was associated with AF64A induced toxicity, and since Ch protected the cells

from cytotoxicity, it would seem likely that Ch might protect the cells from DNA damage induced by AF64A. Therefore DNA damage was assayed at varying concentrations of AF64A (25, 50, and 100 uM for LAN2 and L1210; 250 and 500 uM for A1235) and Ch (10^{-6} M to 10^{-2} M for LAN2 and A1235; 2.15 x 10^{-5} M to 2.15 x 10^{-3} M Ch for L1210).

E. CHAT ACTIVITY STUDIES

AF64A has been shown to inhibit ChAT activity in various preparations such as crude homogenates of rat brain (Rylett and Colhoun, 1979); rat dorsal hippocampus (Mantione et al., 1983); cultured neuroblastoma NG108-15 cells (Sandberg et al., 1985); primary neuronal cultures from chick embryos (Davies et al., 1986); fetal rat brain reaggregate cultures (Atterwill et al., 1986); chick retina (Millar et al., 1987); and various regions of rat brain (Leventer et al., 1987). ChAT activity was measured according to the method of McCaman and Hunt (1965) with modifications by Goldberg, et al. (1969) and Fonnum (1970). Cells were harvested by scraping with a rubber policeman and frozen at 20° C for no more than 2 weeks. Cells were thawed and then homogenized with a Dounce homogenizer (pestle A) using approximately 1.0 ml 75 mM phosphate buffer (pH 7.4, 4° C)/100 ug weight of tissue. Protein levels were measured using the BioRad Protein Assay, which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 when binding to protein occurs (Biorad Instruction Manual, p.1). The reaction mixture (10 ul), containing 75 mM sodium phosphate buffer at pH 7.4, 5 mM Ch, 300 mM NaCl, 20 mM $MgCl_2$, 1 mM eserine sulphate, 0.025% bovine serum

albumin and ^{14}C-acetyl coenzyme A, was reacted with 10 ul of cell

extract for 30 minutes at 38° C. Reaction time was increased from 30 to

60 minutes to increase the amount of product in some of the assays. By

increasing the reaction time, product increased and discrepancies

between control and experimental groups were easier to detect because in

some cases experimental groups were at or just above background. The

reaction was stopped by the addition of 150 ul of 75 mg/ml

tetraphenylboron in 3-heptanone. ACh is soluble in the organic layer.

100 ul of the organic layer was removed and assayed for radioactivity.

The cells were exposed to AF64A for 1, 12 and 24 hours and assayed for

ChAT activity at varying intervals following exposure.

CHAPTER IV

RESULTS

A. COLONY FORMING ASSAYS

1. Survival studies

The cytotoxic effects of AF64A were studied using colony formation assays. These assays measure the ability of cells to replicate and form colonies following exposure to the drug. AF64A produced a dose dependent cell kill in LAN2 cells, a human, cholinergic neuroblastoma cell line (Figure 2A). AF64A was cytotoxic to LAN2 cells regardless of whether they were treated in MEM medium or HBSS. The cells were slightly less sensitive to AF64A when treated in MEM medium, but there was still a dose dependent increase in cytotoxicity. A two log cell kill (1% survival) was produced at an approximate dose of 70 uM in HBSS. An approximate dose of 87.5 uM was required in MEM media to generate a 2 log cell kill. A one hour exposure to 100 uM AF64A produced nearly a 3 log cell kill (0.00231 $\pm$ 0.00076) in HBSS and about a 2.5 log cell kill (0.0055 $\pm$ 0.0023) in MEM media.

A1235 cells were much more resistent to AF64A when compared to the LAN2 cell line (Figure 2B). In order to achieve a one log cell kill, such as that which was achieved at a dose of less than 50 uM with a one hour exposure in the LAN2 cell line, a dose of 500 uM for 2 hours was required in the A1235 cell line (Figure 2B). A one hour exposure to
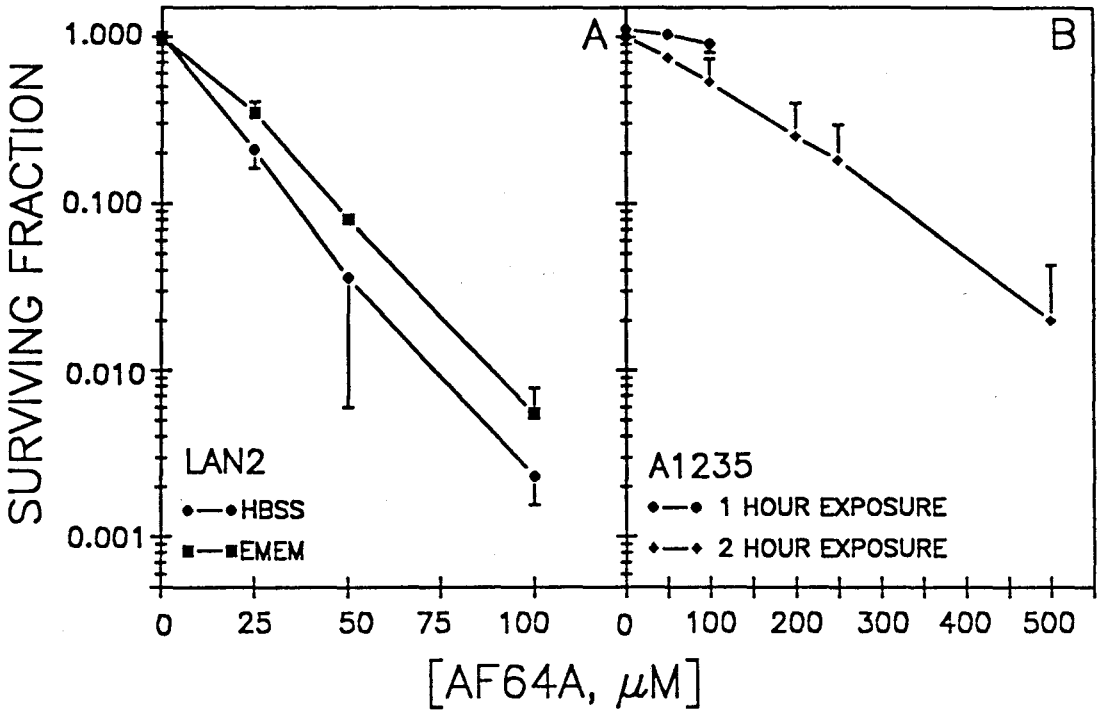
FIGURE 2. A comparison of the cytotoxicity of AF64A in LAN2 (left panel) and A1235 (right panel) cells. LAN2 cells were exposed to AF64A for 1 hour in either MEM medium or HBSS. A1235 cells were exposed to AF64A for either 1 or 2 hours in HBSS. Note the difference in the concentration of AF64A on the X axis. The points represent the mean ± S.D. of at least 3 independent experiments.

100 uM AF64A produced little cytotoxicity, with greater than 90% of the cells surviving treatment. However, with a 2 hour exposure to 100 uM AF64A only 50% of the cells survived treatment.

Figure 3 shows the results of treating mouse leukemia L1210 cells with AF64A. In the initial studies, cells were treated in RPMI 1630 media, while later studies were repeated in HBSS without Ch. AF64A produced a dose dependent increase in cytotoxicity whether treated in HBSS or RPMI 1630 media. However, when treated in HBSS containing no Ch, particularly at the lower doses, the cells appeared to have a greater sensitivity to AF64A. A 50% cell kill was achieved at an approximate dose of 12.5 uM in HBSS without Ch and 30 uM AF64A in RPMI 1630 media with Ch and FCS (2.15 x 10-5 M Ch).

A comparison of the cell kill in the 3 cell lines when exposed to 25, 50 and 100 uM AF64A in HBSS without Ch for 1 hour is shown in Figure 4. AF64A produced very little cell kill in A1235 at these concentrations for this duration of exposure. LAN2 and L1210 cells were about equally sensitive to the drug, with about a 3 log cell kill produced at the highest concentration of AF64A in both cell lines.

2. Choline protection of AF64A induced cytotoxicity

Ch had been shown previously to protect chick embryo primary neuronal cell cultures from the toxic effects of AF64A (Davies et al., 1986). Therefore Ch was used during AF64A exposure to determine whether a protective effect could be obtained in colony formation assays. The plating efficiency of the LAN2 cells increased with increasing Ch concentration. Comparison of LAN2 cells treated in HBSS

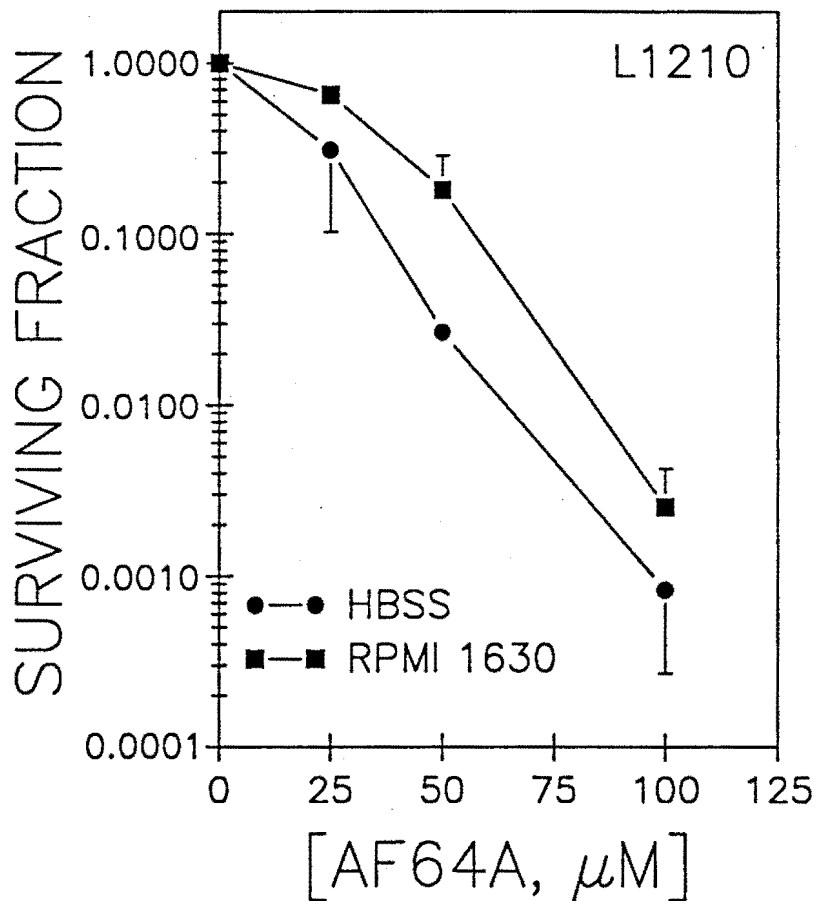FIGURE 3. A comparison of the survival of L1210 cells exposed to AF64A for one hour in either HBSS minus Ch or RPMI 1630 medium. The points represent the mean ± S.D. of at least 3 independent experiments.
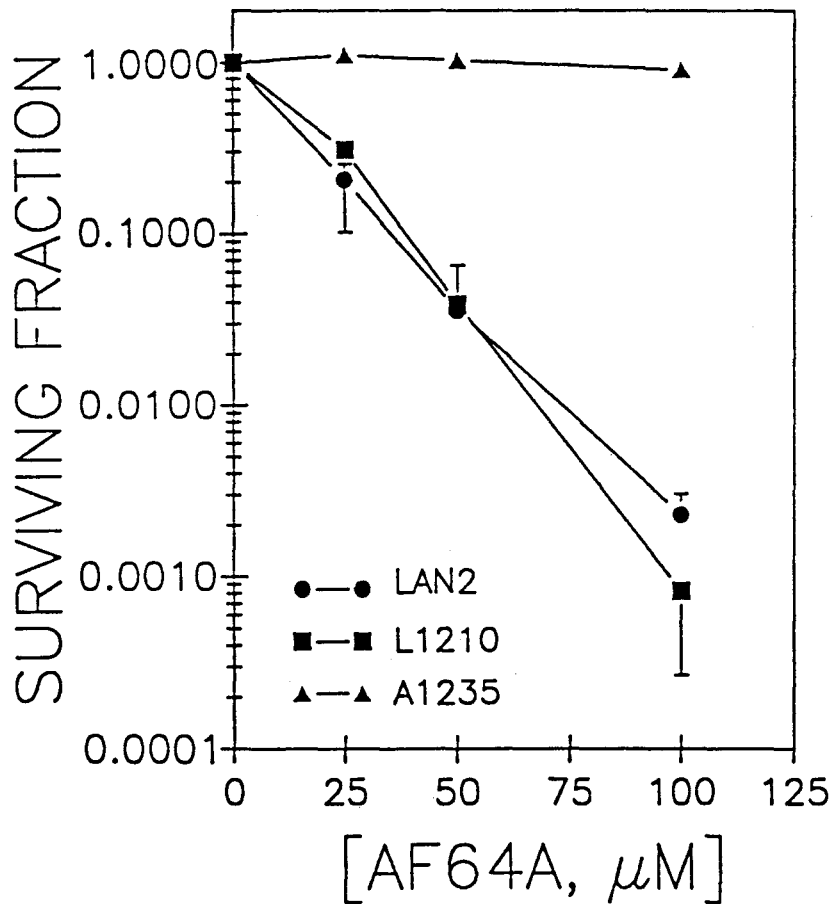
FIGURE 4. A comparison of the cytotoxicity induced by AF64A following one hour of exposure to the three different cell lines in HBSS. The points represent the mean ± S.D. of at least 3 independent experiments.

only and of cells treated in HBSS with 10^{-5} M Ch, showed that there was very little difference in the cytotoxicity produced by AF64A (Figure 5). However, 10^{-4} M Ch had a slight protective effect, preventing about a 1 log cell kill. At a Ch concentration of 10^{-3} M, approximately 50% of the cells survived to form colonies when exposed to 100 uM AF64A, and at a Ch concentration of 10^{-2} M about 75% survived to form colonies when exposed to 100 uM AF64A.

Results in L1210 cells were very similar to the results of the LAN2 studies (Figure 6). A dose of 2.15 x 10^{-5} M Ch had only a slight protective effect. A significant protective effect was seen already at Ch concentrations of 2.15 x 10^{-4} M. A Ch concentration of 2.15 x 10^{-3} M showed nearly complete protection of the L1210 cells from the toxicity of AF64A such that 78% of the cells survived to form colonies at 100 uM AF64A and nearly 100% survived exposure to lower AF64A concentrations.

B. DNA ALKALINE ELUTION STUDIES

1. Measurements of DNA single strand breaks

Following exposure to AF64A for 1 hour, there was a dose dependent increase in the rate of DNA alkaline elution in all cell lines examined, indicating an increased number of DNA single strand breaks (SSB). When measured by this technique, X-irradiation induced random strand breaks in DNA, which produced linear elution profiles when plotted on log-log paper (Figure 7A). When cells, LAN2 in this case, were treated with AF64A, there was also an increase in the rate of alkaline elution (Figure 7B). The shape of the elution profiles varied among experiments, but in general the shape of the majority of elution
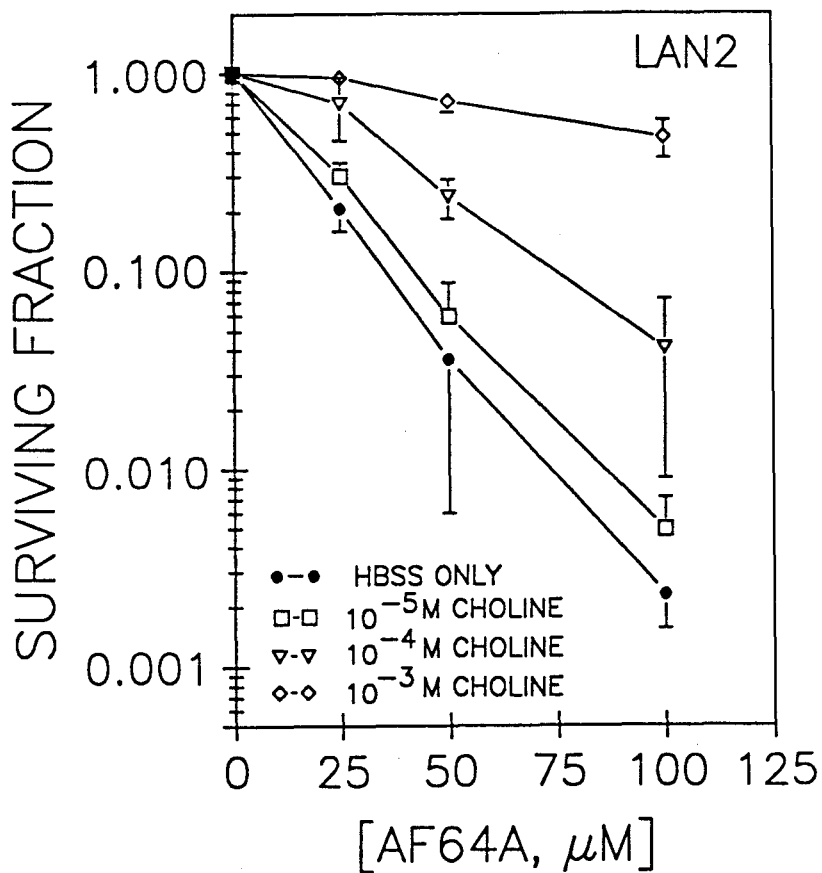
FIGURE 5. The effect of varying the Ch concentration of the medium during drug exposure. LAN2 cells were preincubated with Ch prior to AF64A exposure. The same concentration of Ch was then present during drug treatment. The points represent the mean ± S.D. of at least 3 independent experiments.
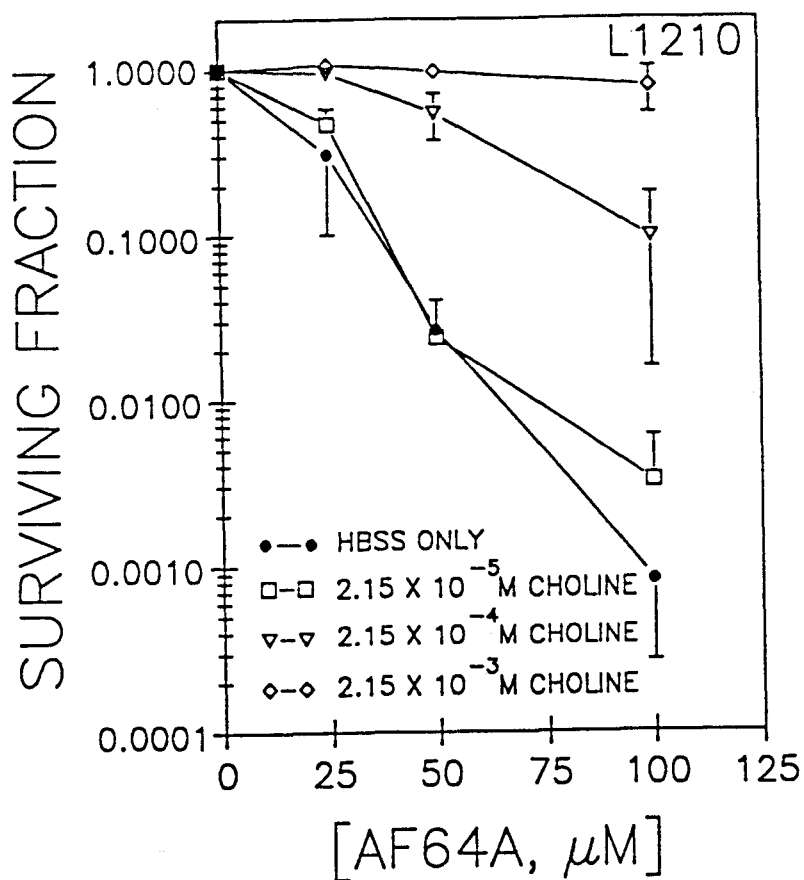
FIGURE 6. The effect of varying the Ch concentration of the medium during drug exposure. L1210 cells were preincubated with Ch prior to AF64A exposure. The same concentration of Ch was then present during drug treatment. The points represent the mean ± S.D. of at least 3 independent experiments.
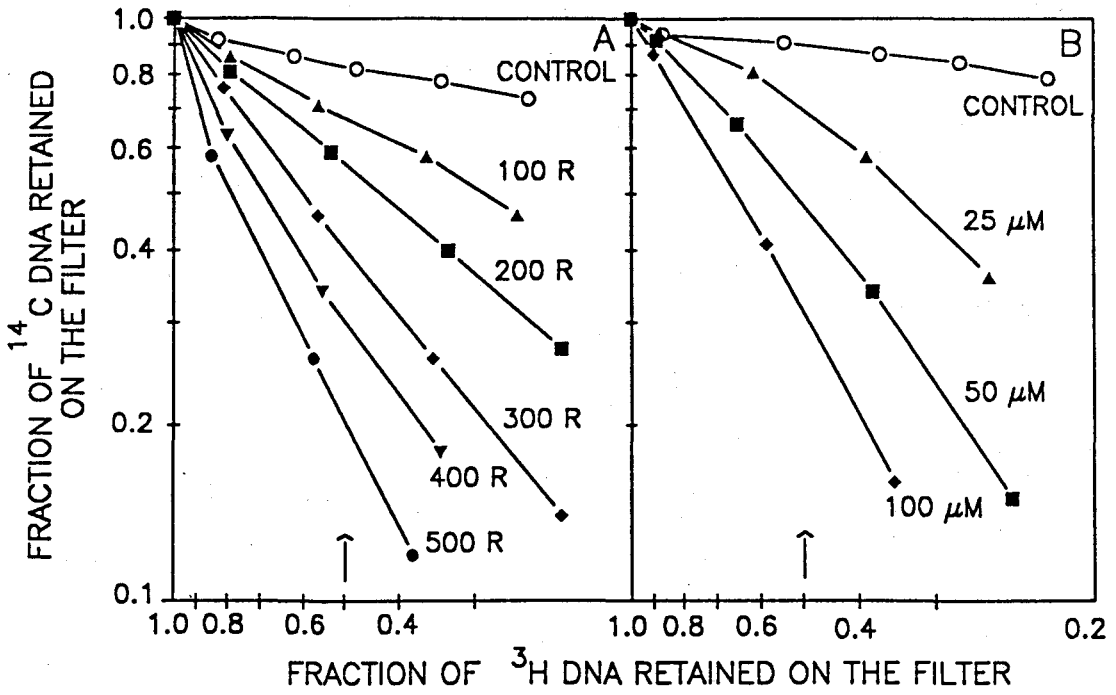
FIGURE 7. Measurement of DNA alkaline elution rate following exposure to X-irradiation (A) or AF64A (B) in LAN2 cells. Increasing elution rate indicates an increase in the amount of strand breaks. The arrow indicates 50% retention of the internal standard DNA. This was the value used to calculate the strand break frequency in terms of rad equivalents.

profiles remained linear through 50% elution of the internal standard DNA (Figure 7B). Therefore, the DNA SSB frequency was determined from the retention of experimental DNA when 50% of the internal standard DNA remained on the filter. Alkali can generate breaks at apurinic sites or in response to chemical lesions of the DNA bases; therefore, it should be noted that although the strand break frequency is calculated in rad equivalents, the breaks may not be frank breaks as seen with radiation, but may be a manifestation of the DNA alkaline elution technique.

Comparison of the breaks in rad equivalents, induced in the three cell lines by AF64A treatment, is shown in Figure 8. At a given molar concentration, AF64A induced the highest frequency of breaks in both the LAN2 and L1210 cell lines. A one hour exposure to 100 uM AF64A produced break frequencies equivalent to 400 Rad in LAN2 and 457 Rad in L1210 cells. In contrast, AF64A produced relatively low frequencies of breaks in the A1235 cell line. In fact, a 2 hour exposure to 500 uM AF64A, produced only 275 rad equivalents (Figure 9). Markedly fewer strand breaks were found at lower doses, and shorter exposure times, of AF64A in the A1235 cell line.

2. Repair of DNA damage

The next series of experiments was performed to study the ability of the cell to repair the SSB induced by AF64A. Strand breaks caused by radiation were very rapidly repaired in LAN2 cells as shown in Figure 10. This was also true for L1210 cells (not shown). Approximately half of the breaks induced by X-irradiation were repaired within the first 7.5 minutes, and nearly complete repair occurred within the first hour.
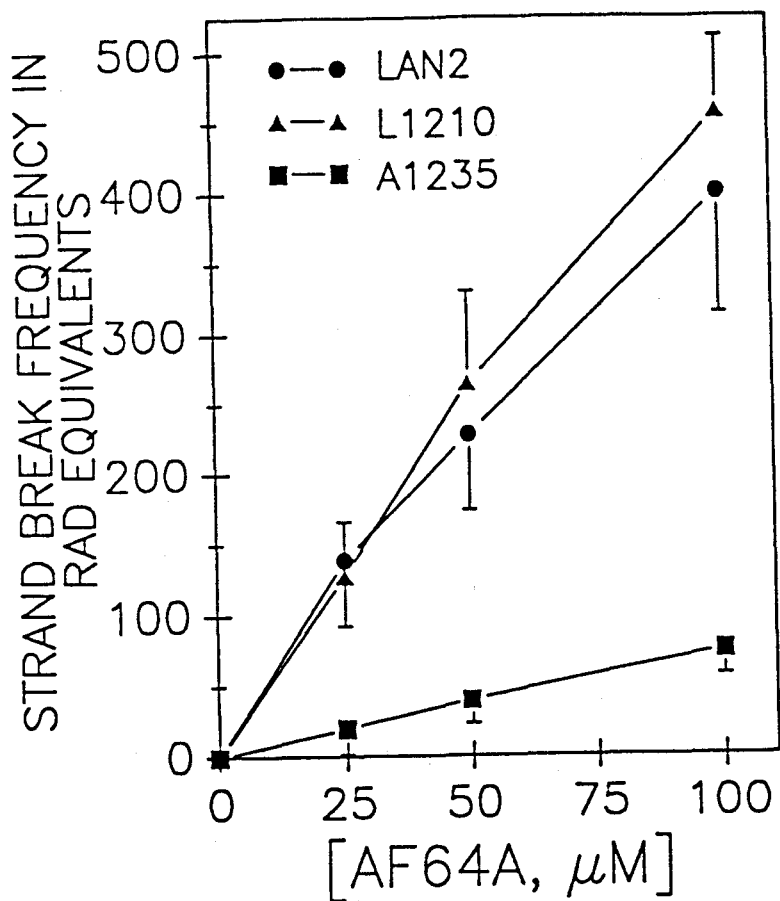
FIGURE 8. A comparison of the strand break frequency generated by AF64A
in 3 different cell lines.   Cells were exposed to AF64A for one hour in
HBSS and alkaline elution was performed immediately following removal of
the drug.   The points represent means $\pm$ S.D. of 3 independent
experiments.
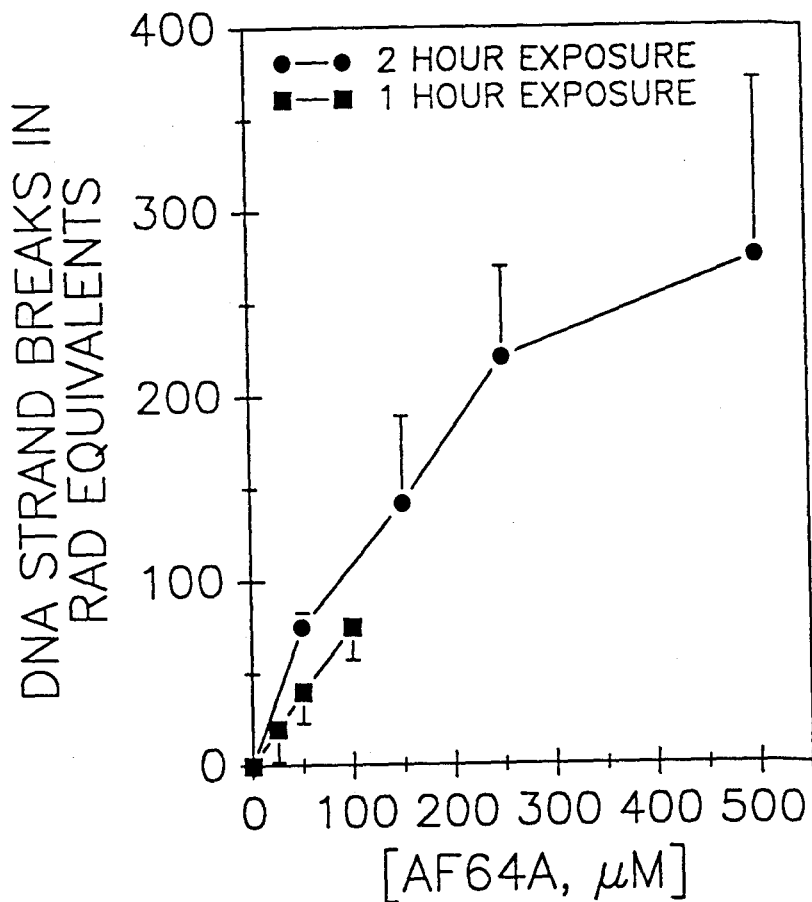
FIGURE 9. The effect of longer exposure on the elution kinetics of A1235 cells. A1235 cells were exposed to AF64A for 1 or 2 hours and alkaline elution assay was conducted immediately following removal of drug. The points represent means ± S.D. of 3 independent experiments.
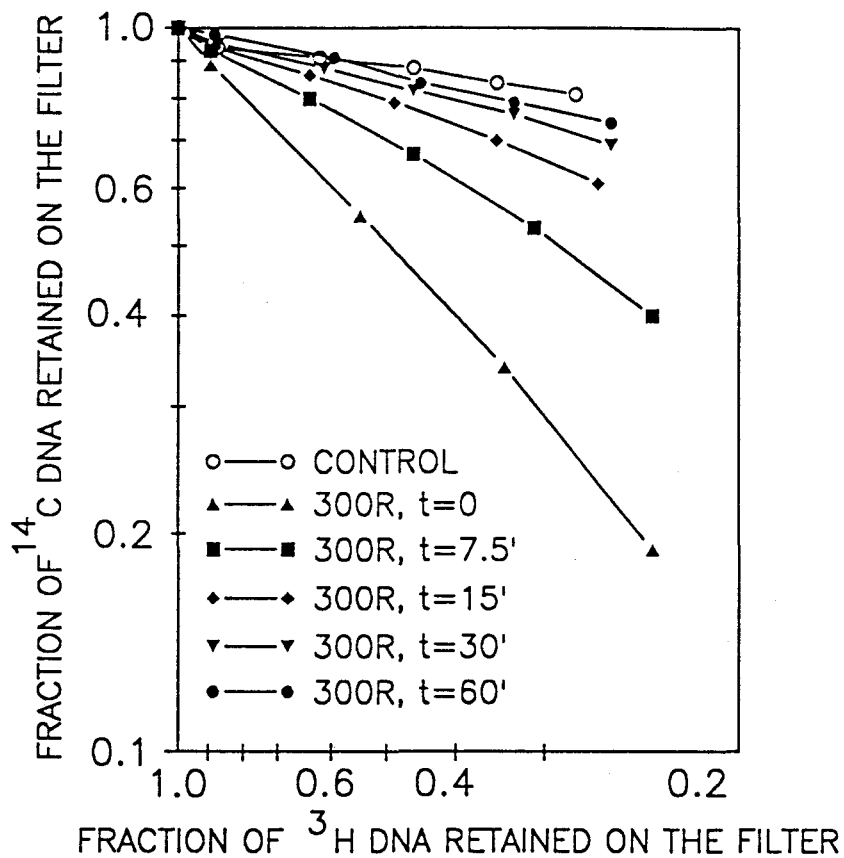
FIGURE 10. The effect of time following exposure to X irradiation on the rate of DNA alkaline elution. LAN2 cells were exposed to 300 R at 0° C and were eluted immediately (t=0) or incubated at 37° C and eluted at various intervals. By 60 minutes elution rates had almost returned to control values.

This contrasts strongly with repair of the breaks produced by AF64A. Representative elution profiles for the 3 cell lines are shown in Figures 11 (LAN2), 12 (A1235), and 13 (L1210). From a comparison of Figures 10 through 13, it can be seen that repair of the damage induced by the drug required a longer period of time than repair of radiation-induced breaks, regardless of the cell line.

LAN2 cells exhibited partial repair of DNA damage by 24 hours (Figure 14). About 50% of the breaks were repaired by 6 hours. Persistent breaks were, however, still obvious at 24 hours at all doses of AF64A. The strand breaks were reduced from approximately 411 Rad equivalents to 107 Rad equivalents by 24 hours following exposure to 100 uM AF64A, indicating repair of about 74% of the DNA damage.

In order to achieve enough breaks to study repair, it was necessary to expose the A1235 cells to higher doses of AF64A for longer periods of time than the LAN2 cells. The SSB frequency produced by AF64A was 275 rad equivalents following a 2 hour exposure to 500 uM in A1235 cells (Figure 15). At 6 hours the frequency of SSB decreased to 212 Rad equivalents and at 12 hours to 133 Rad equivalents. At 24 hours there were still persistent SSB with a frequency equivalent to 94 Rads or about 66% of the damage repaired.

In L1210 cells (Figure 16), exposure to 100 uM AF64A for one hour generated SSB approximately equivalent to 373 Rads when measured immediately following removal of drug. At 6 hours the number of SSB had been reduced to approximately 233 radiation equivalents, and at 12 and 24 hours the number of breaks remained at about 120 radiation equivalents or about 68 percent of the damage repaired. However by 24

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FIGURE 11. The effect of time following exposure to AF64A on the rate of DNA alkaline elution in LAN2 cells. All cells were exposed to AF64A for one hour and then incubated in the absence of drug for 0 hours (upper left); 6 hours (upper right), 12 hours (lower left) or 24 hours (lower right).
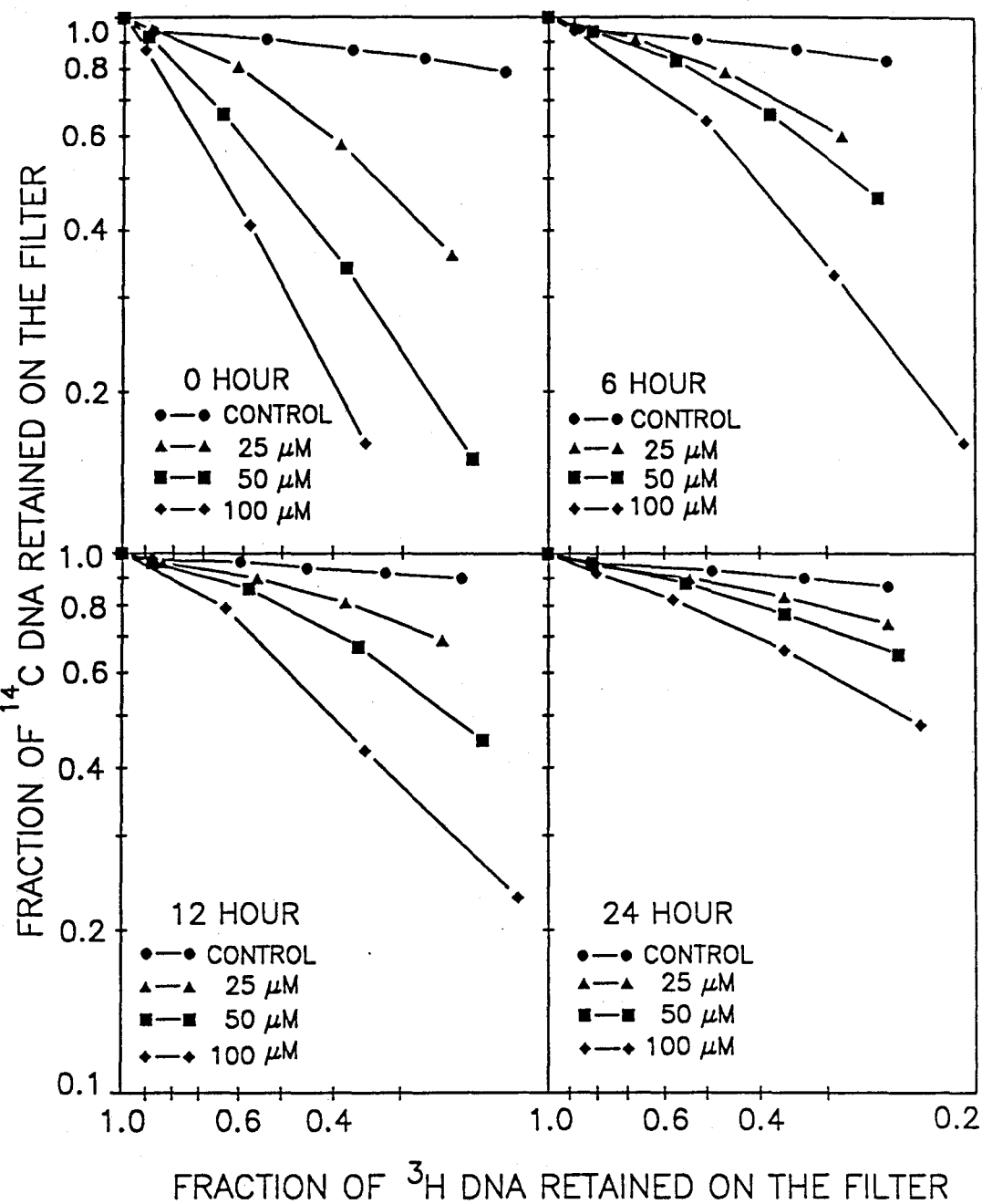
FIGURE 12. The effect of time following exposure to AF64A on the rate of DNA alkaline elution in A1235 cells. All cells were exposed to AF64A for two hours and then incubated in the absence of drug for 0 hours (upper left), 6 hours (upper right), 12 hours (lower left) or 24 hours (lower right).
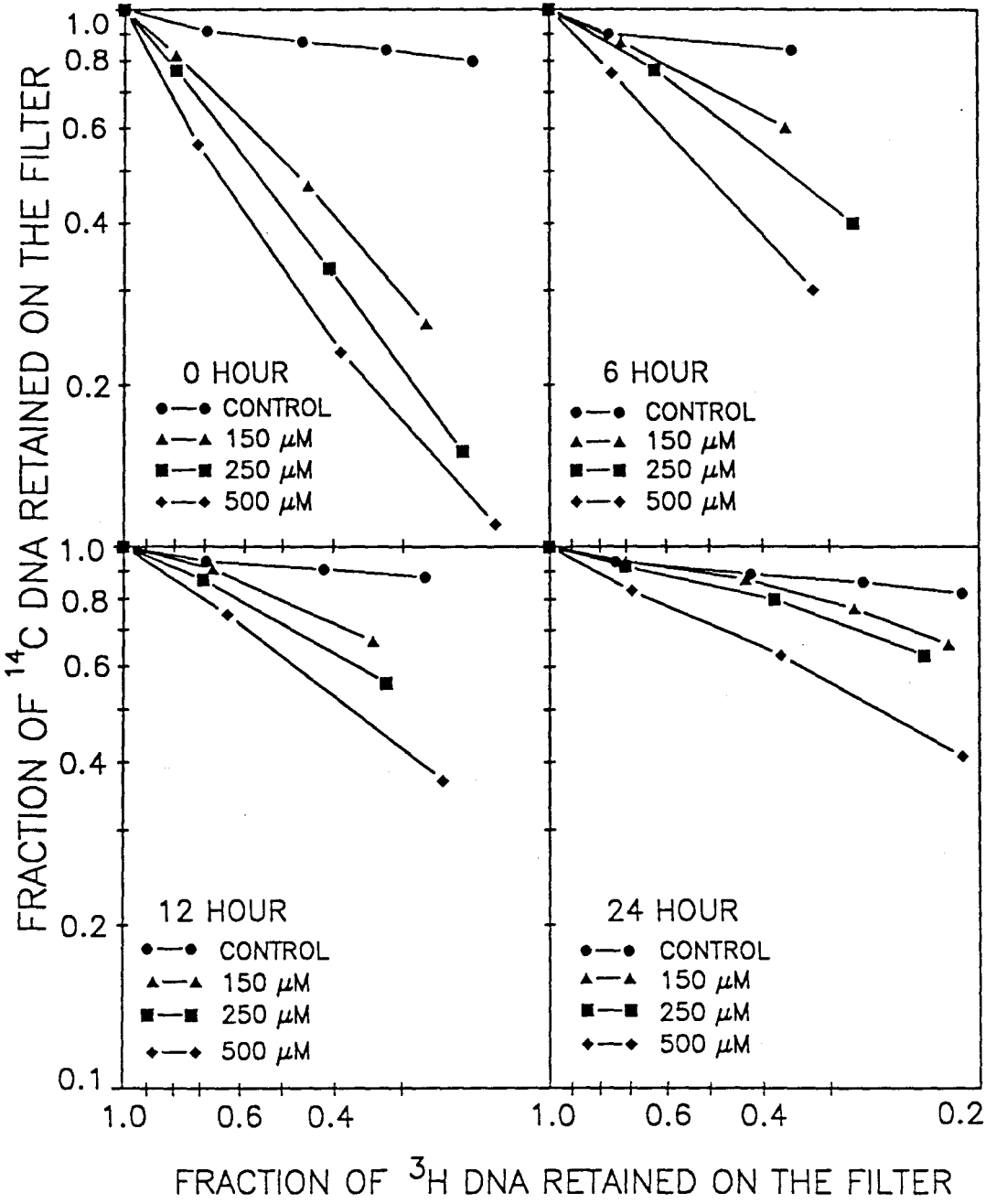
FIGURE 13. The effect of time following exposure to AF64A on the rate of DNA alkaline elution in L1210 cells. All cells were exposed to AF64A for one hour and then incubated in the absence of drug for 0 hours (upper left), 6 hours (upper right), 12 hours (lower left) or 24 hours (lower right).
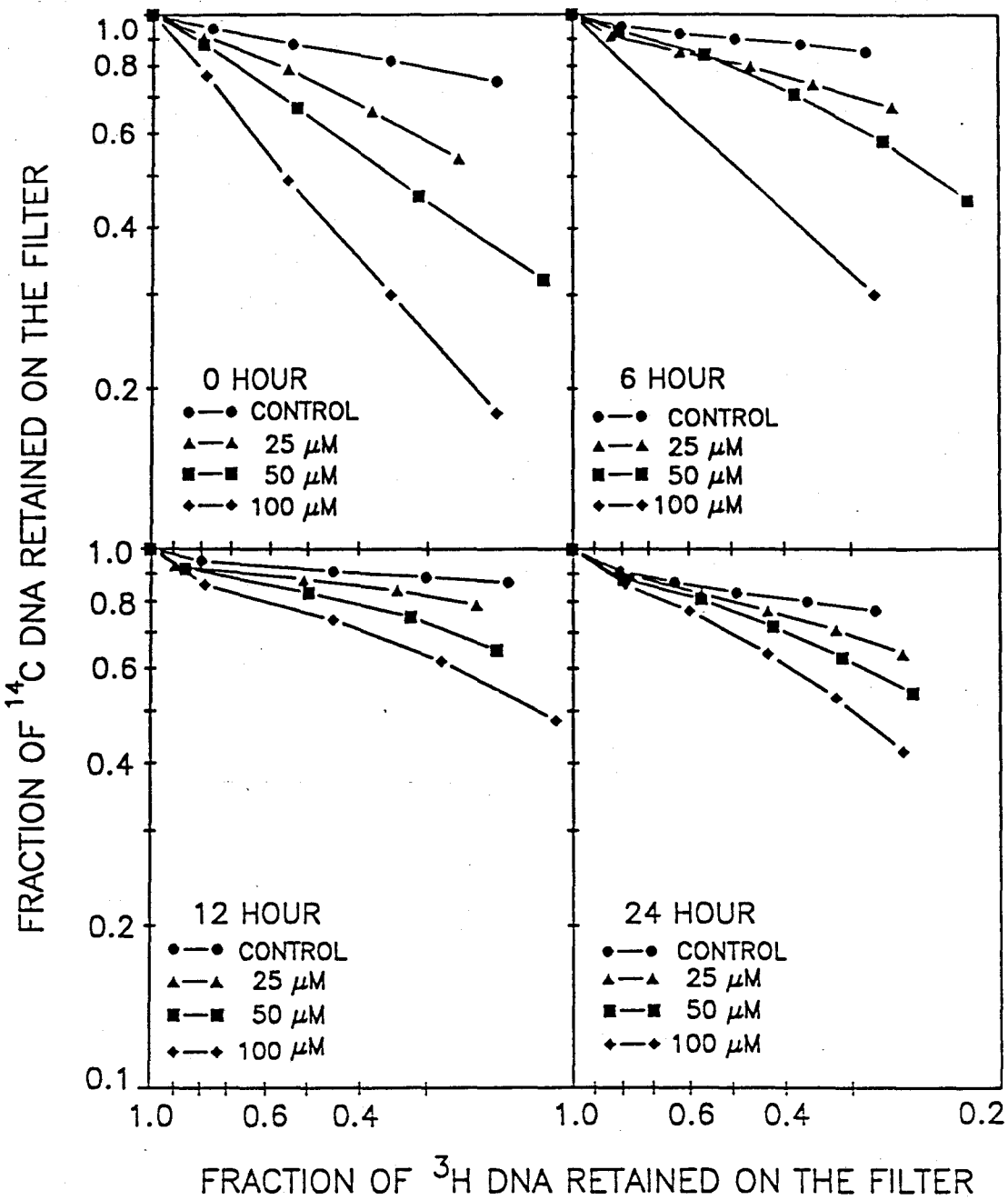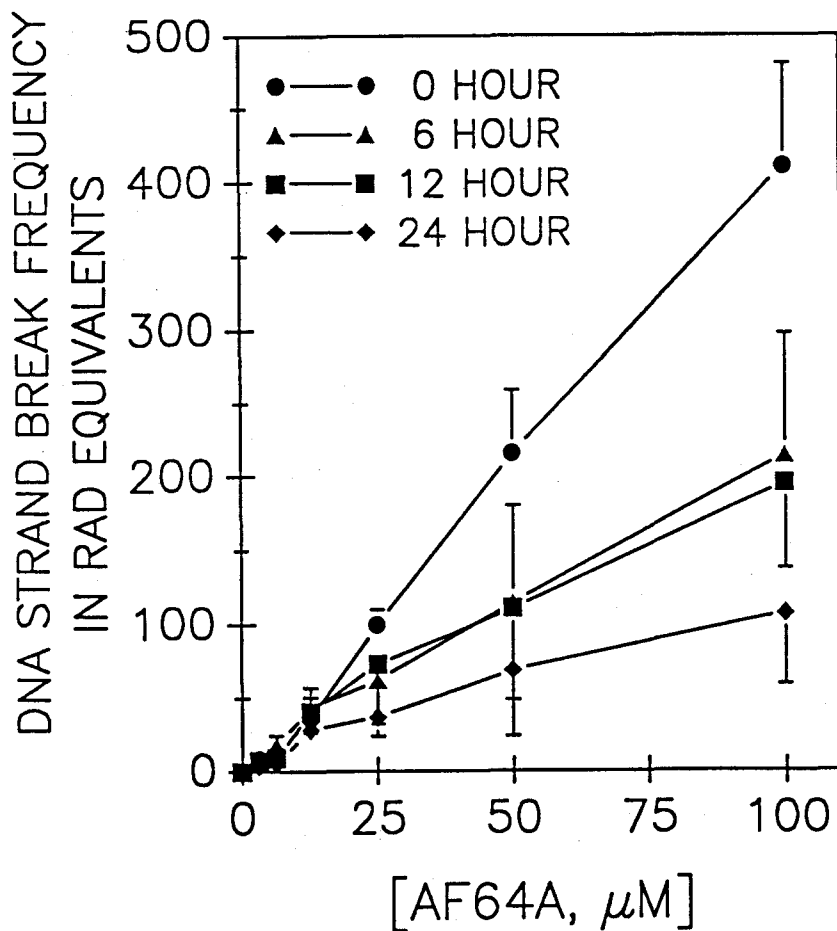
FIGURE 14. The effect of time following exposure to AF64A on DNA strand break frequency in rad equivalents in LAN2 cells. Cells were exposed to AF64A for one hour at 37° in HBSS and then incubated in the absence of drug for 0, 6, 12 or 24 hours. The points represent the mean ± S.D. of 3 independent determinations.
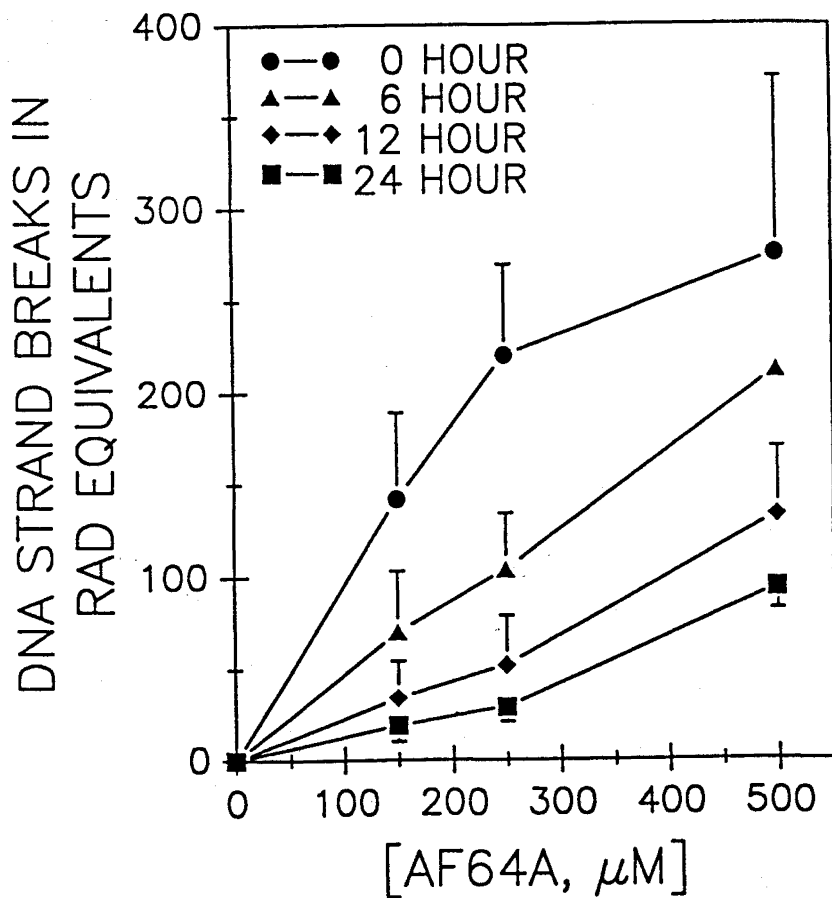
FIGURE 15. The effect of time following exposure to AF64A on DNA strand break frequency in rad equivalents in A1235 cells. Cells were exposed to AF64A for two hours at 37° in HBSS and then incubated in the absence of drug for 0, 6, 12 or 24 hours. The points represent the mean ± S.D. of 3 independent determinations.
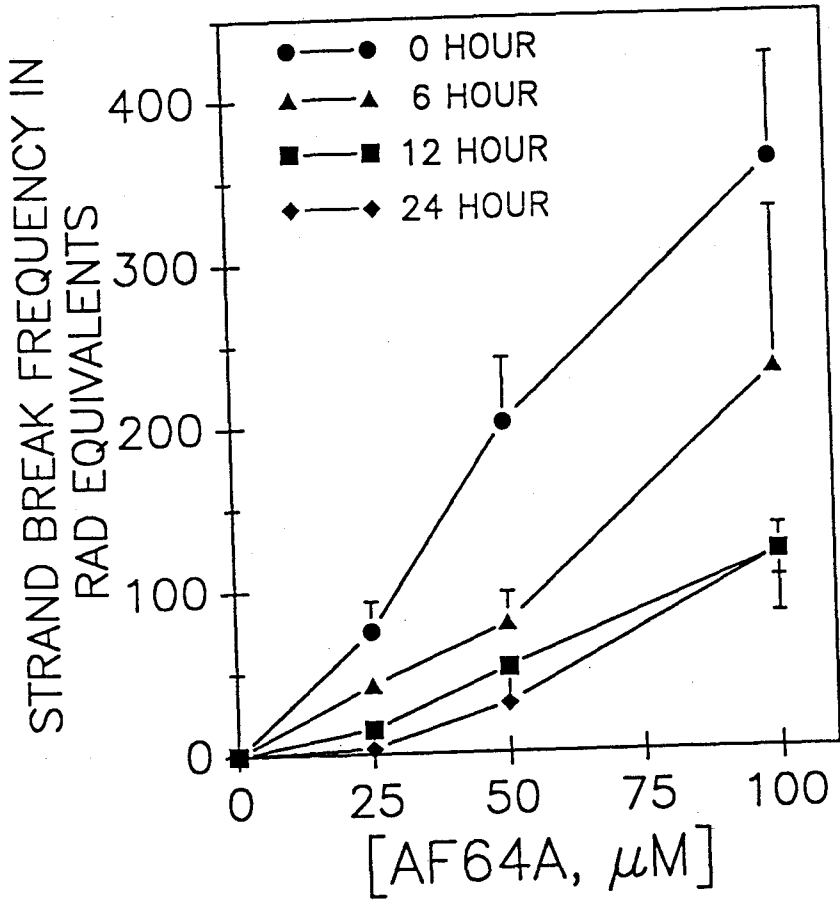
FIGURE 16. The effect of time following exposure to AF64A on DNA strand break frequency in rad equivalents in L1210 cells. Cells were exposed to AF64A for one hour at 37° in HBSS and then incubated in the absence of drug for 0, 6, 12 or 24 hours. The points represent the mean ± S.D. of 3 independent determinations.

hours the DNA of cells exposed to lower doses of AF64A was almost
completely repaired.

3. Tests for alkali labile lesions

It is known that some monofunctional alkylating agents produce
alkali labile sites which are cleaved at a higher pH (greater than 12.1)
and allow DNA to elute at a faster rate than cells which have not been
exposed to the alkylating agent. The elution profiles of AF64A were
compared to those of methyl methanesulfonate (MMS) and methylnitrosourea
(MNU). MNU and MMS were chosen for comparison because MNU had been
shown to produce alkali labile sites and MMS had not (Wani and
D'Ambrosio, 1986).

The DNA of LAN2 cells exposed MNU showed a considerably faster
elution rate at pH=12.6 than at pH=12.1 (Figure 17A and 17B). When LAN2
cells were exposed to MMS, which is not considered to produce alkali-
labile sites, there was little difference between elution at pH=12.1 or
pH=12.6 (Figure 17C and 17D). In a similar fashion, there was little
difference between elution at pH=12.1 or pH=12.6 following exposure to
AF64A (Figure 18). A comparison of mean break frequency in terms of rad
equivalents among the three cell lines is shown in Figure 19. In each
case there were more breaks in the DNA eluted at pH=12.6. When doses
producing roughly equivalent break frequencies are compared, such as
6.25 uM AF64A and 25 uM MNU, there was on the average, a 17% increase in
the number of breaks in the AF64A treated group and a 67% increase in
the number of breaks in the MNU treated group when eluted at pH=12.6. A
comparable dose of MMS, 50 uM, produced about a 13% increase in the

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FIGURE 17. The effect of pH on the rate of DNA alkaline elution in LAN2 cells. Cells were exposed to MNU for one hour and then eluted at pH 12.1 (top left) or pH 12.6 (top right) or exposed to MMS for one hour and eluted at pH 12.1 (bottom left) or pH 12.6 (bottom right). Increasing the pH of elution greatly increased the rate of alkaline elution in cells treated with MNU but not in cells exposed to MMS.
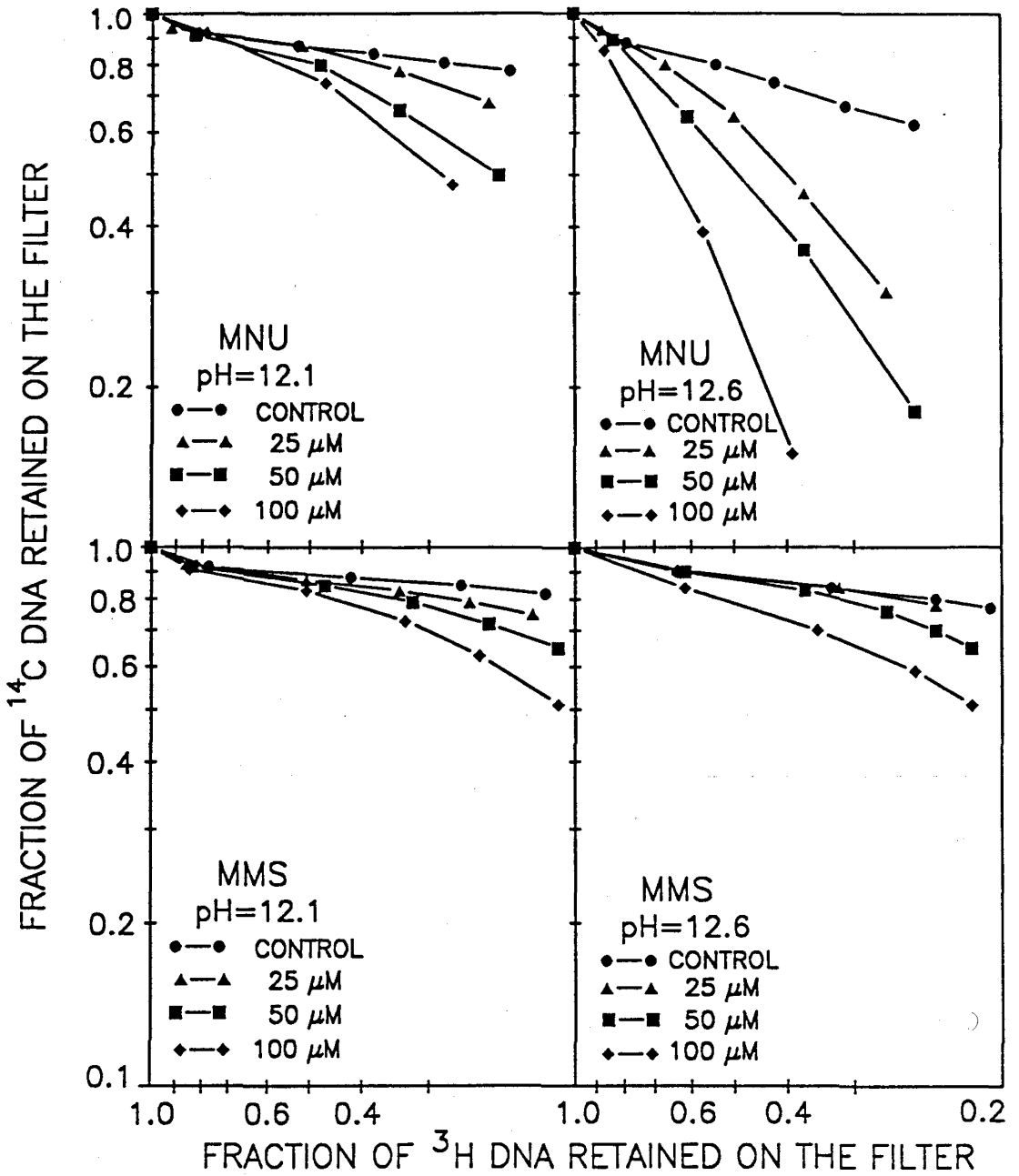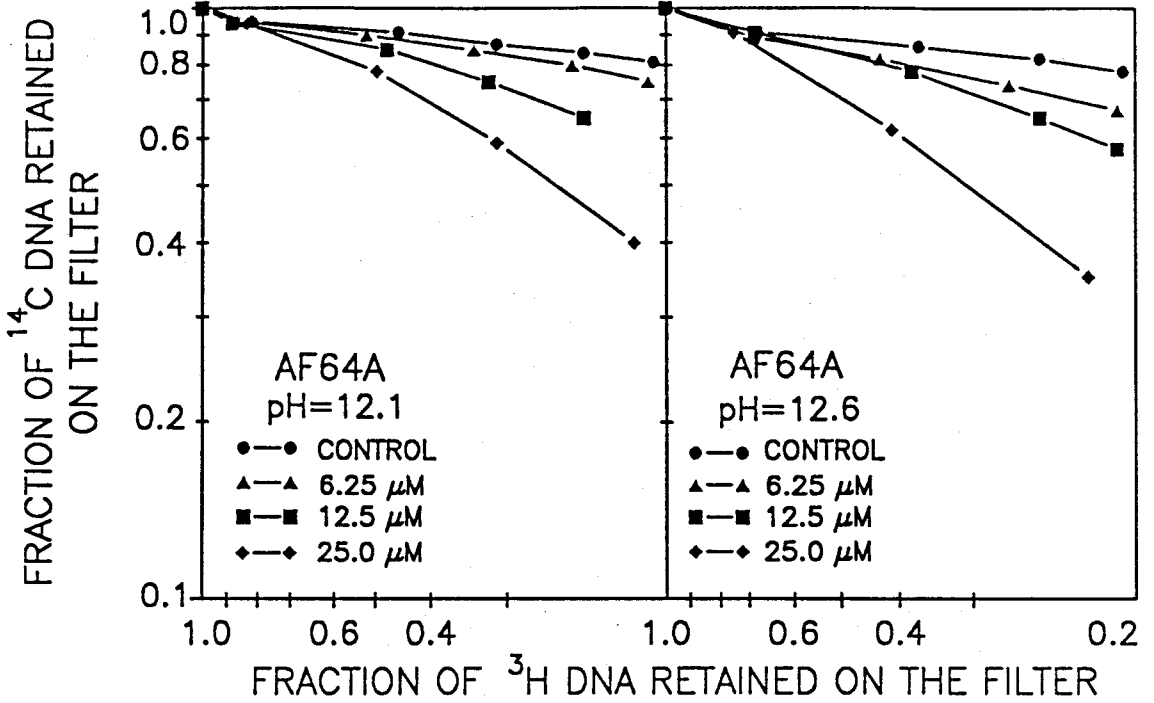
FIGURE 18. The effect of pH on the rate of DNA alkaline elution in LAN2 cells. Cells were exposed to AF64A for one hour and then eluted at pH 12.1 (left panel) or pH 12.6 (right panel). Increasing the pH of elution did not greatly increase the rate of alkaline elution in cells treated with AF64A.

FIGURE 19. A comparison of the strand break frequency in rad equivalents produced by AF64A, MNU and MMS when eluted at pH 12.1 and pH 12.6. LAN2 cells were exposed to the different drugs for one hour in HBSS at 37° C. The lower X axis was used for the AF64A concentrations and the upper X axis was used for the MMS and MNU concentrations. The two different X axes were used for comparison because the lower concentrations of AF64A produced a strand break frequency roughly equivalent to higher concentrations of MMS and MNU. The points represent the mean $\pm$ S.D. of 3 independent determinations.
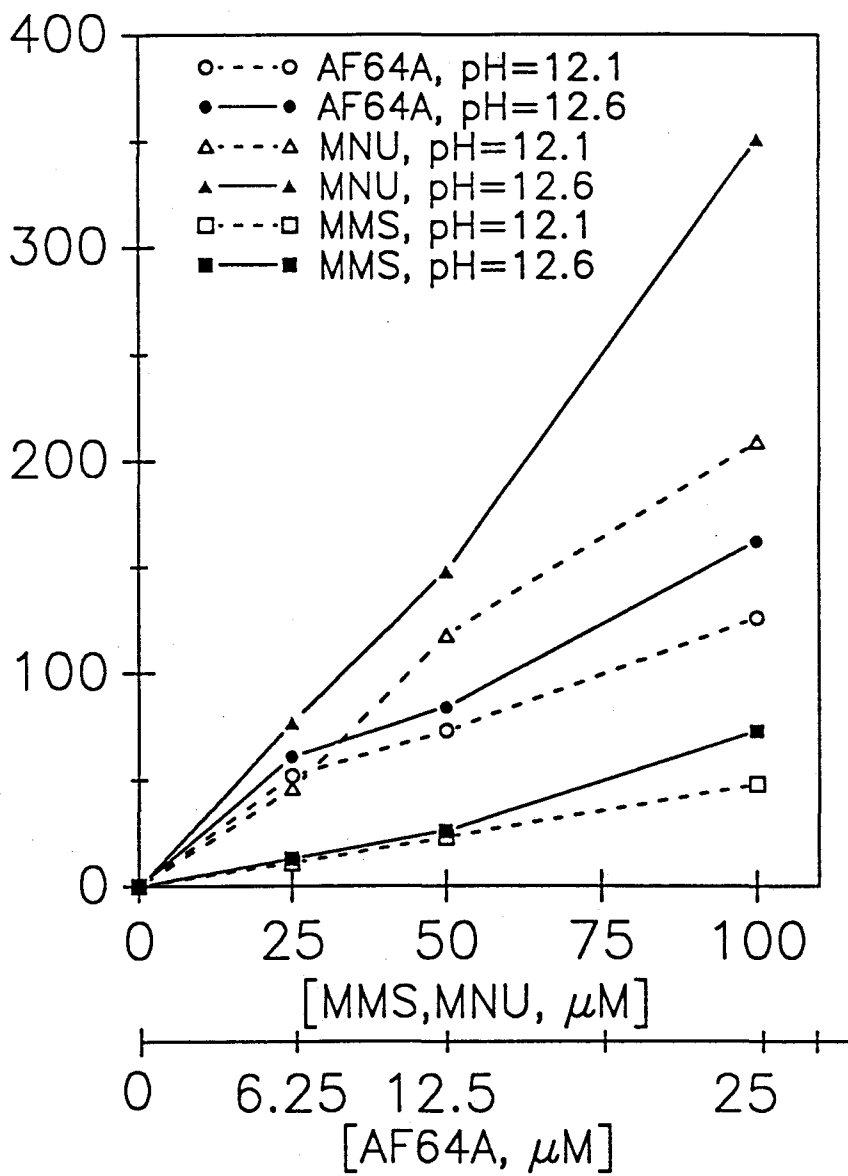
number of breaks on the average. The higher doses tended to produce more alkali labile sites with each of the compounds tested.

4. Choline protection of AF64A-induced DNA damage

Ch had previously been shown to protect cells from cytotoxicity produced by AF64A (Davies et al., 1986). Therefore, similar to the earlier studies on Ch protection of AF64A induced cytotoxicity, the next series of experiments studied the effects of combining AF64A and Ch in the medium, to determine whether Ch could protect against the DNA damage generated by AF64A. In Figure 20, the elution profiles of the LAN2 cell line are shown. Essentially no protection from DNA damage was evident at a Ch concentration of 10^{-5} M Ch. There was an intermediate level of protection when a concentration of 10^{-4} M Ch was used, and a concentration of 10^{-3} M Ch produced nearly complete protection. The results of the mean break frequency in radiation equivalents for LAN2 cells are presented in Figure 21. A dose of 100 uM AF64A in HBSS produced a break frequency equivalent to 355 Rads. A Ch concentration of 10^{-5} M did not reduce the frequency of SSB. 10^{-4} M Ch reduced the breaks generated by 100 uM AF64A to 215 Rad equivalents and 10^{-3} M Ch protected the cells, so that a dose of 100 uM AF64A produced a mean break frequency equivalent to 40 Rads.

Ch protection in A1235 cells was consistent with results generated from the LAN2 cell line (Figure 22). Very little protection was evident at Ch concentrations of 10^{-5} M. Some reduction in breaks was seen at a concentration of 10^{-4} M Ch. Finally, the cells were almost completely protected from DNA damage at a Ch concentration of 10^{-3} M.

FIGURE 20. The effect of Ch concentration on the rate of DNA alkaline elution in LAN2 cells. Cells were exposed to AF64A for one hour in HBSS containing no Ch (top left), 10^{-5} M Ch (top right), 10^{-4} M Ch (bottom left) or 10^{-3} M Ch (bottom right).
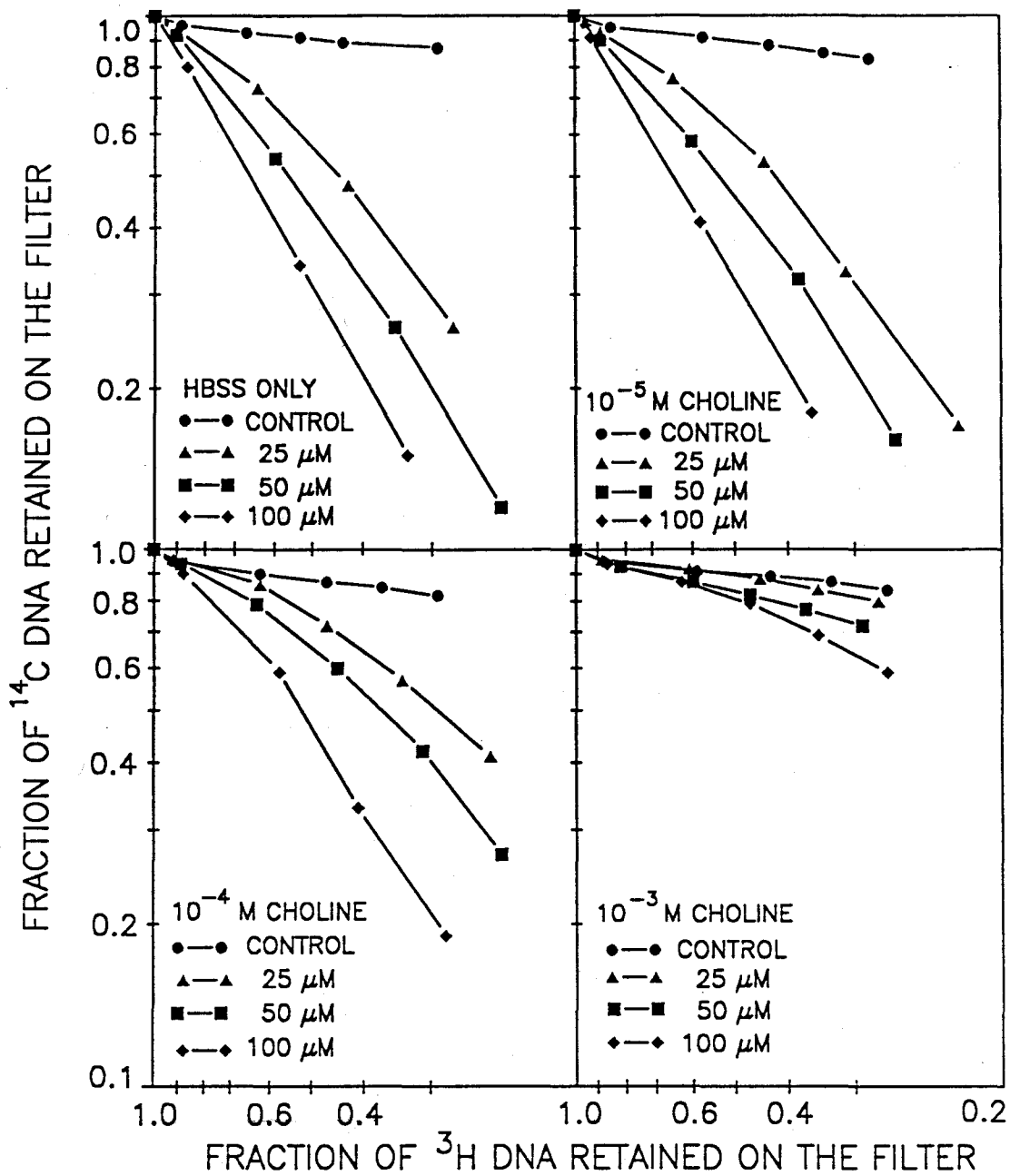
FIGURE 21. The strand break frequency in rad equivalents produced by AF64A when treated concomitantly with various concentrations of Ch. LAN2 cells were pretreated with the concentration of Ch indicated, and then treated with AF64A and the appropriate concentration of Ch in HBSS. Elution immediately followed removal of drug. The points represent the mean ± S.D. of 3 independent determinations.

FIGURE 22. The effect of Ch concentration on the rate of DNA alkaline elution in A1235 cells. Cells were exposed to AF64A for two hours in HBSS containing no Ch (top left), 10^{-5} M Ch (top right), 10^{-4} M Ch (bottom left) or 10^{-3} M Ch (bottom right).
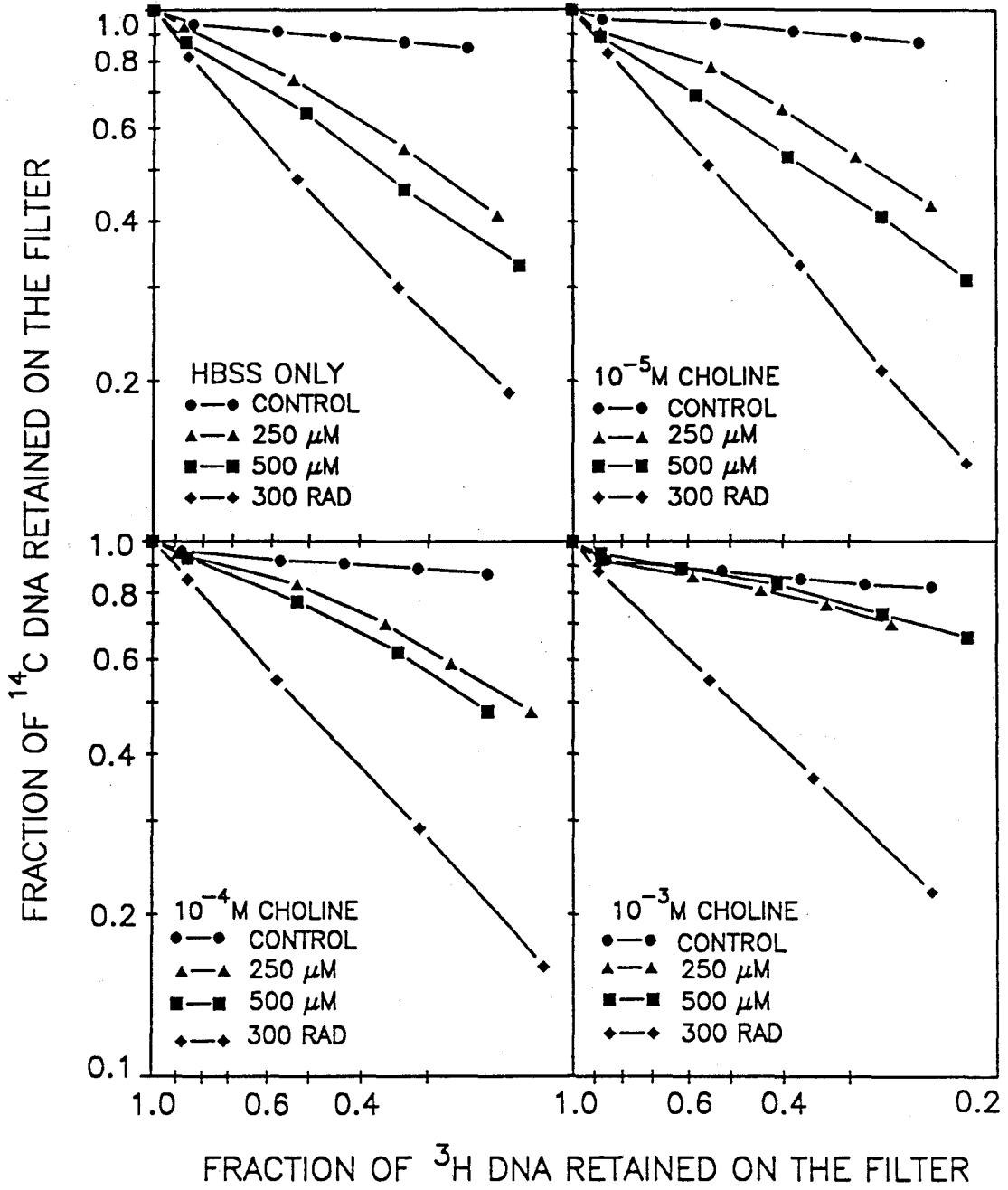
The results of the L1210 studies were similar to the studies involving the other cell lines (Figures 23 and 24). Concentrations of Ch from 2.15×10^{-5} M to 2.15×10^{-3} M were chosen. Figure 24 shows the results of the Ch protection studies in terms of radiation equivalents. There was little difference between the number of breaks in cells treated in HBSS or in those treated in HBSS fortified with 2.15×10^{-5} M Ch. However in cells treated in HBSS fortified with 2.15×10^{-4} M Ch, the number of breaks was considerably reduced from a mean break frequency of over 400 Rad equivalents to 260 Rad equivalents when exposed to 100 uM AF64A. When cells were treated in HBSS containing 2.15×10^{-3} M Ch almost no breaks were seen at lower doses, although there were a few at higher concentrations of AF64A.

C. ASSAY FOR ChAT ACTIVITY IN AF64A TREATED CELLS

The results of the ChAT assays are presented in Figures 25 and 26. There was considerable variability in the absolute ChAT activity among the experiments, therefore ChAT activity is expressed as a percent of control with the control assumed to have 100% activity. When LAN2 cells were treated for one hour at 50 uM or 100 uM there were 20% and 33% reductions in ChAT activity, respectively (Figure 25). These were significantly different from control to the $p<.01$ (**) and $p<.001$ (***) confidence levels using a paired Student t test. With continuous exposures to AF64A for 12 and 24 hours even greater reductions in ChAT activity were seen. Morphologically, cells treated for long exposures were different from control. They had reduced processes and most had detached from the flask. At 12 hours ChAT activity was reduced by 33%

FIGURE 23. The effect of Ch concentration on the rate of DNA alkaline elution in L1210 cells. Cells were exposed to AF64A for one hour in HBSS containing no Ch (top left), 2.15 X 10^{-5} M Ch (top right), 2.15 X 10^{-4} M Ch (bottom left) or 2.15 X 10^{-3} M Ch (bottom right).

FRACTION OF ^{14}C DNA RETAINED ON THE FILTER

FRACTION OF ^{3}H DNA RETAINED ON THE FILTER

HBSS ONLY
●—● CONTROL
▲—▲ 25 μM
■—■ 50 μM
◆—◆ 100 μM

2.15 X 10^{-5}M
CHOLINE
●—● CONTROL
▲—▲ 25 μM
■—■ 50 μM
◆—◆ 100 μM

2.15 X 10^{-4}M
CHOLINE
●—● CONTROL
▲—▲ 25 μM
■—■ 50 μM
◆—◆ 100 μM

2.15 X 10^{-3}M
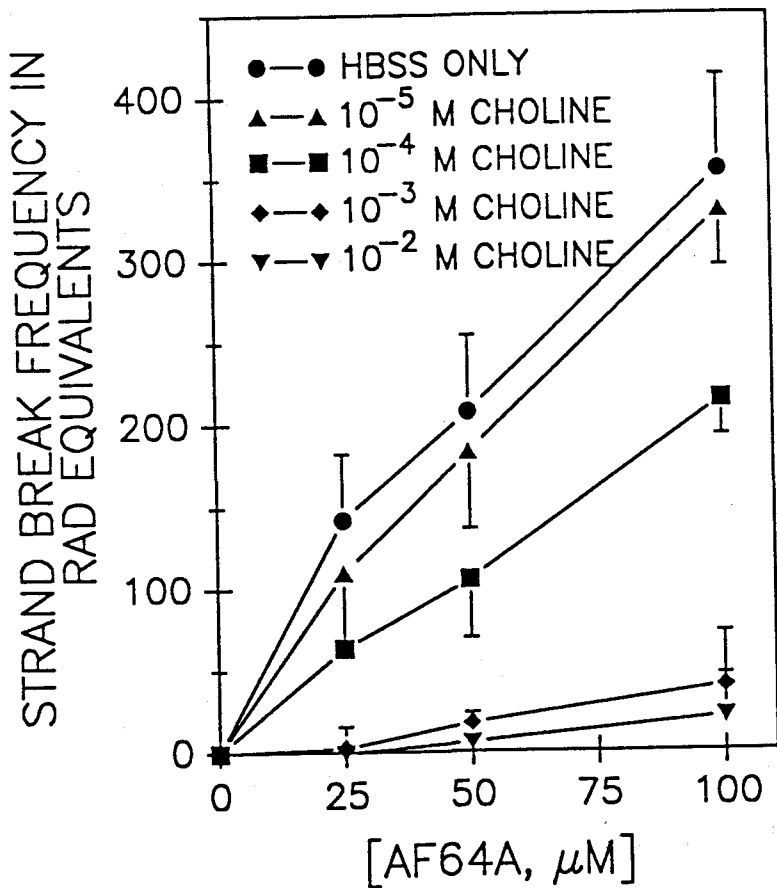CHOLINE
●—● CONTROL
▲—▲ 25 μM
■—■ 50 μM
◆—◆ 100 μM
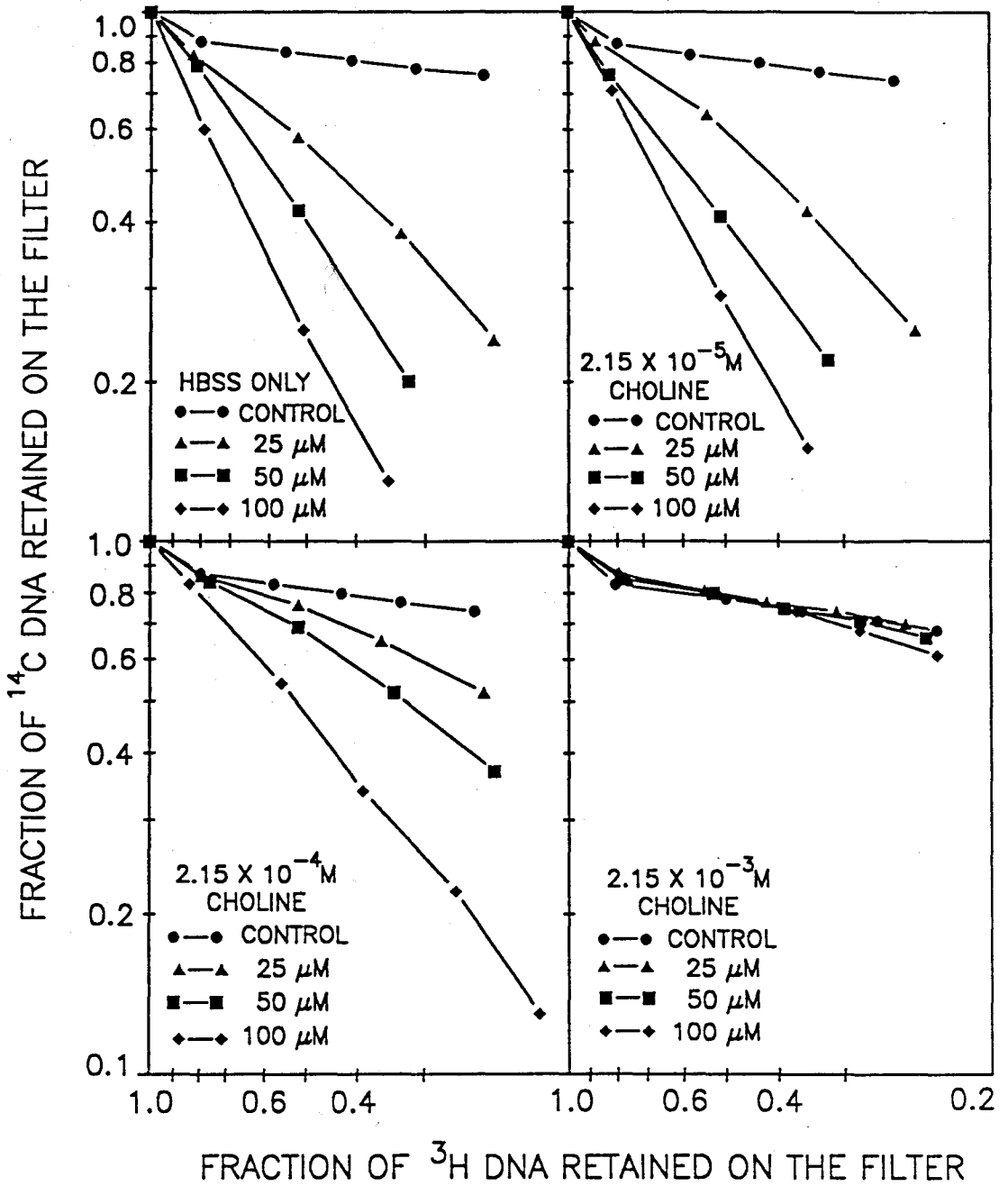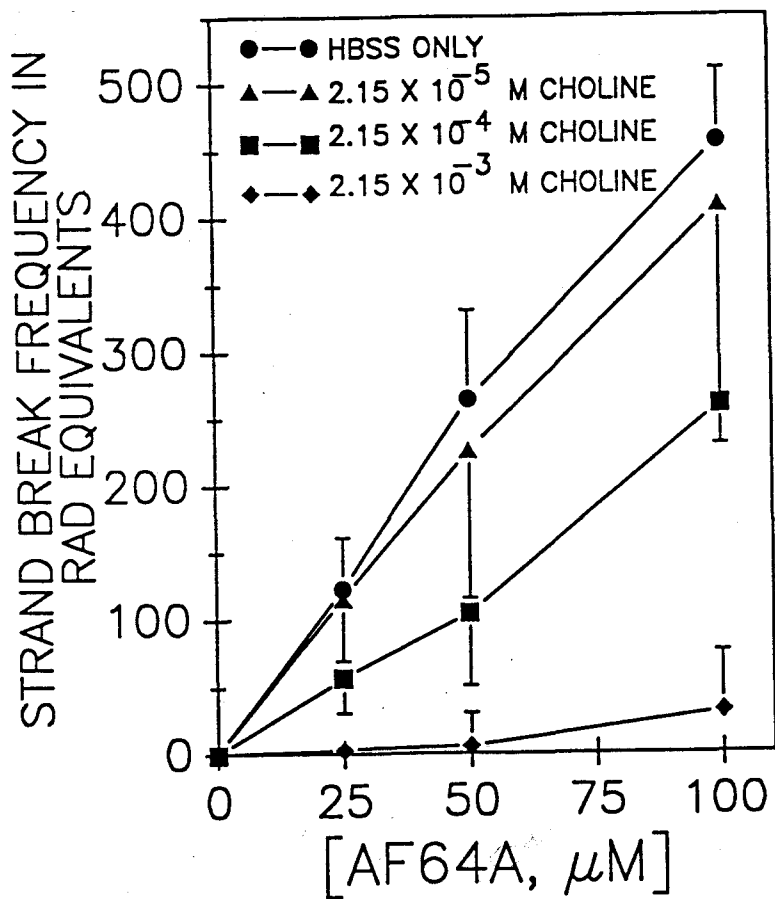
FIGURE 24. The strand break frequency in rad equivalents produced by AF64A when treated concomitantly with various Ch concentrations. L1210 cells were pretreated with the Ch concentration indicated and then treated with AF64A and the appropriate concentration of Ch in HBSS. Elution immediately followed removal of drug. The points represent the mean $\pm$ S.D. of 3 independent determinations.
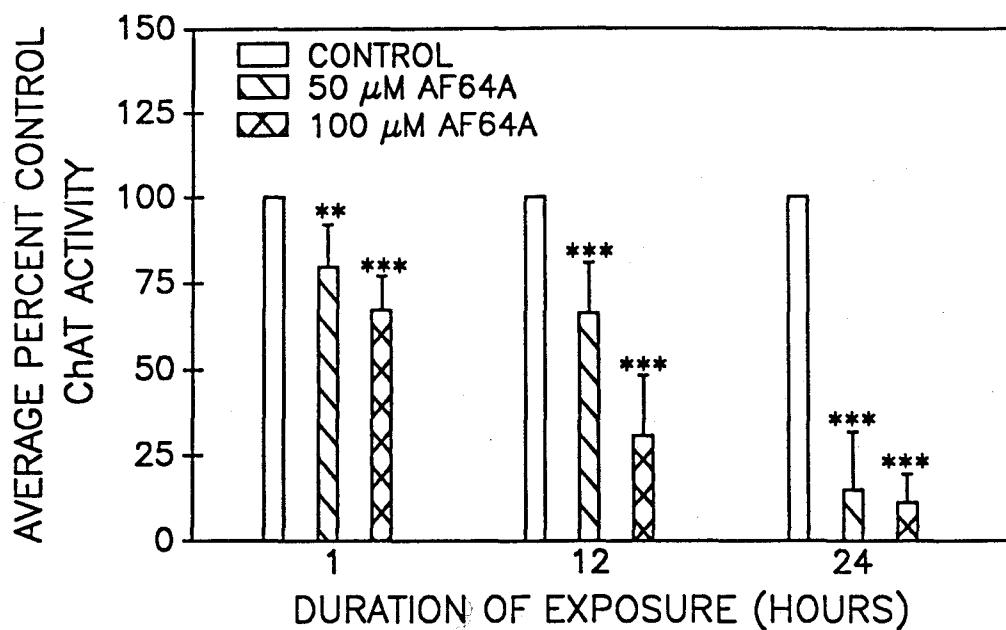
FIGURE 25. The effect of increasing duration of exposure to AF64A on the percent control ChAT activity in LAN2 cells. Cells were exposed to AF64A for 1, 12 or 24 hours in MEM at 37° C. Cells were then harvested and frozen and ChAT activity determined within 2 weeks. The points represent the mean ± S.D. of at least 3 independent determinations. The significance was determined by Students' T test, ** p< .01 and *** p<.001.
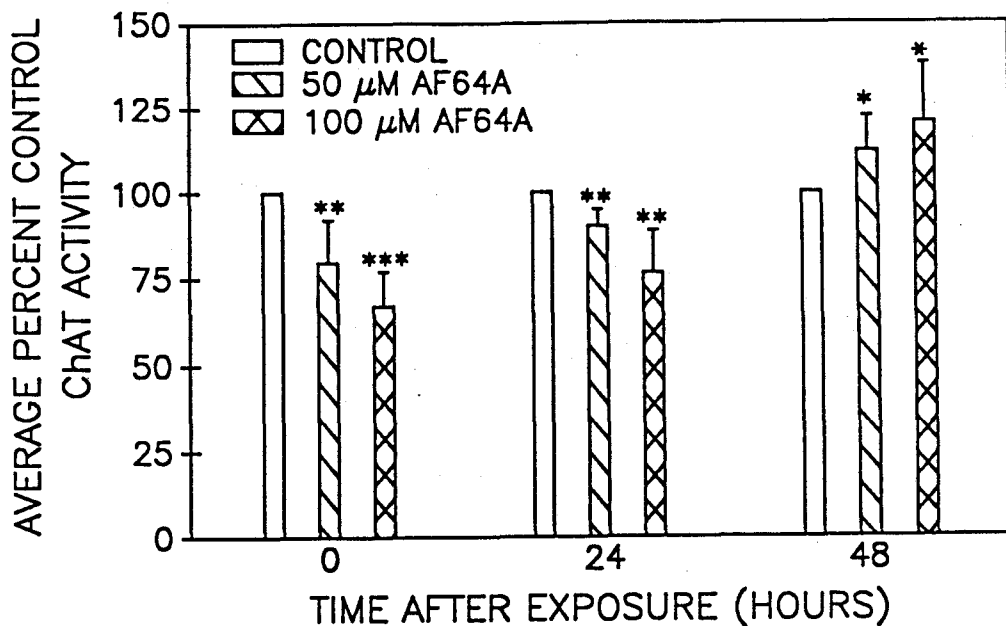
FIGURE 26. The effect of a one hour exposure to AF64A on the percent control ChAT activity in LAN2 cells. Cells were exposed to AF64A for 1 hour in MEM at 37° C then harvested at 0, 24 and 48 hours. Cells were frozen and ChAT activity determined within 2 weeks. The points represent the mean ± S.D. of at least 3 independent determinations. The significance was determined by Students' T test, * p<.05 and ** p< .01.

and 69% in 50 uM and 100 uM AF64A, respectively. At 24 hours activity

was reduced even more, by 85% and 90%. Thus with continuing exposure to

AF64A greater reductions in ChAT activity were seen. When cells were

exposed to 50 or 100 uM AF64A for one hour and then the drug was

removed, ChAT activity remained depressed for 24 hours.   However by 48

hours a small, but significant rebound increase in ChAT activity was

seen p<.05 (*) (Figure 26).

CHAPTER V

DISCUSSION

The purpose of this research was to further elucidate the
mechanisms of action of AF64A. AF64A has previously been shown to be an
inhibitor of HAChT and ChAT (Pittel, et al., 1987; Rylett and Colhoun,
1980), although is unlikely that inhibition of either would account for
the cytotoxic actions of AF64A, since cells which do not contain ChAT or
HAChT are still susceptible to AF64A induced cytotoxicity. Therefore
one goal of this study was to determine what cellular insults might
cause the cytotoxic action of AF64A. The other goal of this study was
to examine the specificity of action of AF64A by quantifying the
cytotoxicity and DNA damage generated by AF64A in one cholinergic and
two non-cholinergic cell lines.

A. CYTOTOXICITY STUDIES

The cytotoxic actions of AF64A were compared by means of colony
formation assays in three different cell lines: LAN2, a human
cholinergic neuroblastoma cell line; A1235, a human non-cholinergic
neuroglioma cell line; and L1210, a rapidly dividing mouse leukemia cell
line frequently used for anti-tumor drug studies. As shown in Figure 4,
AF64A was far more cytotoxic to the L1210 and LAN2 cells than the A1235
cells.

One possible explanation for the differential cytotoxicity between

the cell lines could be that each of the cell lines may be transporting different amounts of AF64A and allowing different amounts of the drug to accumulate inside the cell (Amir et al., 1988). Other workers have shown that nitrogen mustard enters the cell via Ch transport mechanisms (Goldenberg and Vanstone, 1971; Goldenberg, et al., 1971b; Goldenberg and Sinha, 1973). Differences in the rate of transport of nitrogen mustard has been postulated to account for differences in cytotoxicity between cell lines (Wolpert and Ruddon, 1969; Chun, et al., 1969). It has also been suggested that AF64A may be entering the cell via both high and low affinity Ch transport, and that the cells with higher rates of Ch transport would sequester more of the drug (Pittel, et al., 1987; Amir et al., 1988). Therefore, if AF64A does enter the cell via Ch transport mechanisms, the cells with higher rates of Ch transport would take up more of the drug than cells with lower rates of Ch transport.

Further evidence that the difference in Ch transport may be responsible for the differences in cytotoxicity is that Ch had a protective effect on LAN2 and L1210 cells preventing the AF64A induced cytotoxicity (Figures 5 and 6). When Ch was present in the medium in equimolar concentrations with AF64A, it was found to have an intermediate protective effect. When Ch was present at ten times the concentration of AF64A, it was almost completely effective in preventing AF64A induced cytotoxicity. Thus Ch could conceivably exert its protective effect by competing with AF64A for entrance into the cell.

Although, of the cell lines used in this study, the actual rate of Ch transport has only been reported for L1210 cells, it is likely that LAN2 cells would have a higher rate of Ch transport than A1235 cells.

It is also likely that LAN2 and L1210 cells may have a similar rate of Ch transport, if LAN2 cells have transport rates similar to other cholinergic cell lines. The rate of Ch transport has been shown to be approximately 4.5 pmoles/min/200,000 L1210 cells and is linear through at least 20 minutes (Naujokaitis et al., 1984). In a study by Lanks et al. (1974), the rate of Ch transport in a neuroblastoma clone that was cholinergic was shown to be 3.89 pmoles/min/200,000 N18 cells and a non-cholinergic cell line was reported to have a Ch uptake rate of 0.28 pmoles/min/200,000 N1E cells (1974). Massarelli et al. demonstrated that cholinergic neuroblastoma clones (N18) had a higher rate of Ch transport than the non-cholinergic clones (S21) and that Ch uptake increased at a linear rate for at least an hour in the cholinergic cells while transport of Ch in the non-cholinergic cells was saturable (1974a,b).

It is particularly interesting to note that Ch protected both LAN2 and L1210 from AF64A induced cytotoxicity even though only the LAN2 are a cholinergic cell line. This again suggests that it is not damage to enzymes involved in ACh synthesis which are responsible for AF64A induced cytotoxicity, but is due to the consequences of AF64A entering the cell via Ch transport mechanisms. It should also be noted that, although AF64A exhibited differential cytotoxicity, it can become toxic to resistant cells, i.e. A1235, if given in higher doses for a longer period of time. This would be expected if differences in Ch transport rate accounted for differences in cytotoxicity and if the transport of the mustard via Ch transport mechanisms was linear with time.

B. ALKALINE ELUTION STUDIES

1. DNA Strand breaks

The breaks induced by AF64A could be attributed to two separate causes. The first potential explanation for an increase in strand breaks following exposure to AF64A, is alkylation of the DNA by the aziridinium portion of the molecule. Depending on the site of alkylation, the mustard can induce lesions in the DNA which appear as single strand breaks when the cell is exposed to alkali. Compounds structurally very similar to AF64A have been shown to preferentially alkylate the N^7 position on deoxyguanosine (Price et al., 1968). Utilizing a modification of the Maxam and Gilbert DNA sequencing technique, AF64A has been shown to alkylate the N^7 position of guanine in DNA exposed to AF64A in vitro (Futscher and Erickson, personal communication). When guanine is alkylated at the N^7 position it leads to a labilization of the imidazole ring, and ultimately to ring opening (Chetsanga et al, 1982). This, in turn, can weaken the glycosidic linkage, leading to a spontaneous depurination in vitro (Price et al., 1968). The base can also be released by a glycosylase, possibly the FAPy glycosylase which has been shown to remove formamidopyrimidine from DNA both in vitro and in vivo (Margison and Pegg, 1981; Chetsanga et al., 1982). These apurinic sites can produce strand breaks when exposed to alkali (Bayley et al., 1961; Jones et al., 1968; Wani and D'Ambrosia, 1986).

The second possible explanation for the origin of AF64A induced strand breaks is a depletion of Ch from the cell. It has been shown that hepatic cells in rats fed Ch deficient diets for 3-5 days develop

an increase in the single strand break frequency of the DNA when measured by alkaline elution (Rushmore et al., 1986). These strand breaks are not due to cell death and are repaired if Ch is supplied to the cells. It was postulated that when the cells are starved for Ch, it leads to lipid peroxidation from the nuclear membrane generating free radicals which damage the DNA causing strand breaks (Rushmore et al., 1986). If AF64A is alkylating Ch transport sites, inhibiting Ch transport and depleting the cells of Ch, perhaps Ch deprivation, could lead to the generation of strand breaks.

This second hypothesis may account for some, but probably not all, the strand breaks generated by AF64A. In the work by Rushmore rats did not show signs of strand breaks until 3 days of Ch deprivation, and numerous breaks were observed only after 5 days of Ch deprivation. In the present study, all cell lines showed strand breaks after only one hour of exposure to AF64A. Also, the majority of the strand breaks were repaired within 24 hours, even though Ch transport is likely to continue to be inhibited at the concentrations of AF64A studied (Pittel et al., 1987). However, even control cells treated in Ch free media have a lower plating efficiency and slightly more strand breaks than cells treated in Ch supplemented media. Therefore, Ch deprivation may account for some but probably not all of the strand breaks produced by AF64A. The more likely cause of strand breaks is probably AF64A binding to the DNA base and causing either spontaneous depurination, or removal of the damaged base through glycosylases, which lead to strand breaks when measured by DNA alkaline elution.

2. Repair of Strand Breaks

The rate of repair of strand breaks was studied in these cells as well. In all 3 cell lines the number of breaks in the DNA was studied at 0, 6, 12 and 24 hours after removal of the drug. The rate of repair of the strand breaks was very slow when compared to the repair of strand breaks generated by ionizing radiation. Approximately half the breaks generated by 300 R X-irradiation were repaired within the first 7.5 minutes and nearly all the breaks were repaired within the first hour (Figure 10). On the other hand, less than one half of the lesions generated by AF64A were repaired at 6 hours, and were not completely repaired even at 24 hours. It is also interesting to note that the LD_{50} for X-irradiation was approximately 300 rad in L1210 cells (data not shown). In these same cells the LD_{50} for AF64A was less than 25 uM. This concentration of AF64A produced strand breaks roughly equivalent to 90 rad in L1210 cells. This suggests that the DNA lesions generated by AF64A are different in nature and far more toxic to the cells than lesions produced by X-irradiation. The breaks produced by X-irradiation were reported to be "frank breaks" (Birnboim, 1986) repaired rapidly by the action of DNA ligase (Stamoto and Hu, 1987), whereas chemical lesions have been reported to be most likely repaired more slowly by the action of several enzymes (Smith, 1987). It has been reported that a damaged base can be removed by a FAPy glycosylase (Margison and Pegg, 1981; Chetsanga et al., 1982). Following removal of the damaged base several different repair mechanisms might occur. A base might be inserted into the apurinic site by the action of a DNA insertase (Deutsch and Linn, 1979 a,b). Alternatively, the DNA may undergo a

short patch and long patch repair which involves the action of apurinic endonucleases (Snyder and Regan, 1982; Erixson, 1985; Smith, 1987) and subsequently DNA polymerases and ligases (Erixson, 1985).

The differences observed in cytotoxicity among the cell lines does not appear to be due to differences in the ability to repair damage. In each of the cell lines the highest dose of AF64A tested produced about a 2 to 3 log cell kill, i.e. a surviving fraction of 1.0% to 0.1%, which correlated with an initial number of strand breaks which varied between approximately 300 to 400 rad equivalents. When the initial number of breaks was about 400 rad equivalents, the number of strand breaks remaining in the DNA at 24 hours was roughly equivalent to 100 rads, so that each cell line was able to repair about 75% of the damage by 24 hours. Therefore, the differences observed in cytotoxicity appear to relate to the initial amount of DNA damage by AF64A, rather than deficiencies in the ability of the different cell lines to repair the DNA damage.

3. Tests for alkali lability

It is known that alkali will cleave a base-free site in DNA, such as would be produced by a glycosylase (Wani and D'Ambrosia, 1986). It was therefore of interest to determine whether the strand breaks generated by AF64A were alkali labile. The elution profiles of AF64A were compared to those of MNU and MMS. Nitrosoureas are known to alkylate a number of sites in DNA, such as the phosphate backbone and guanine N^7 and O^6. This class of drugs produced alkali labile sites, presumably because of alkylation of the phosphate backbone (Wani and

D'Ambrosio, 1986). MMS, on the other hand, primarily alkylated the N^7

position on guanine (Lown and McLaughlin, 1979). The elution profiles

of AF64A more closely resemble those of MMS (Figures 17-19). Elution of

DNA of cells treated with AF64A and MMS at pH 12.6 as compared to

elution at pH 12.1, produced about 20% more strand breaks on the

average. At pH 12.6, MNU produced approximately 40% more breaks (Figure

19). Apurinic sites are hydrolyzed more readily at a lower pH than the

phosphotriesters (PTE) formed by alkylation of the phosphate backbone

(Wani and D'Ambrosia, 1986). MNU alkylates both the base and the

phosphate backbone and both of these lesions can lead to alkali labile

sites (Wani and D'Ambrosia, 1986). However a greater percentage of the

apurinic sites will generate strand breaks at pH 12.1 than PTE, which

will not be labile at this pH. When the PTE are exposed to a pH 12.6, a

greater percentage will result in strand breaks, accounting for the far

greater percentage of alkali labile sites seen in cells exposed to MNU

when compared to cells treated with MMS and AF64A.

4. Choline protection of AF64A induced breaks

Consistent with the cytotoxicity data were the data showing Ch

prevention of strand breaks generated by AF64A. In all 3 cell lines

concentrations of 10^{-4} M Ch were able to partially protect the cell from

AF64A induced breaks at doses of 100 uM in LAN2 and L1210 and 500 uM in

A1235. Concentrations of 1 mM Ch almost completely protected the cells

from these same doses of AF64A. The results were the same, regardless

of whether the cells were treated concomitantly with Ch and AF64A, or if

they were pretreated with Ch for one hour prior to exposure to AF64A and

Ch. These results correlate well with the cytotoxicity data, in which an intermediate cell kill was observed with 10^{-4} M Ch and little cytotoxicity was observed at 10^{-3} M Ch. This, again, suggests that AF64A is being taken up by Ch transport mechanisms, and that increasing uptake of AF64A leads to increasing numbers of strand breaks.

C. CORRELATION BETWEEN CYTOTOXICITY AND STRAND BREAKS

There was a strong correlation between the cytotoxicity of AF64A in terms of log cell kill and DNA single strand break frequency in terms of radiation equivalents (Figure 27). When the log cell kill and strand break frequency were plotted against each other at equimolar concentrations of AF64A for all three cell lines, using all data which could be compared, ie. cytotoxicity, strand break elutions, and Ch protection studies for all three cell lines, a regression line was generated with a correlation coefficient of r=0.93 (Figure 27). This implies a causal relationship between DNA damage and cell death, and is in agreement with others who have found that strand breaks, particularly in critical regions involved in replicon initiation, can lead to cell death (Elkind, 1985). Others have shown that apurinic sites inhibit the action of DNA polymerase and thus presumably lead to cell death (Boiteaux and Laval, 1983). Still other have shown that apurinic sites can lead to mutations and presumably cell death (Shearman and Loeb, 1979; Kunkel, 1984). Thus, the lesions produced by AF64A would presumably lead to mutations or inhibition of DNA synthesis and therefore cell death.
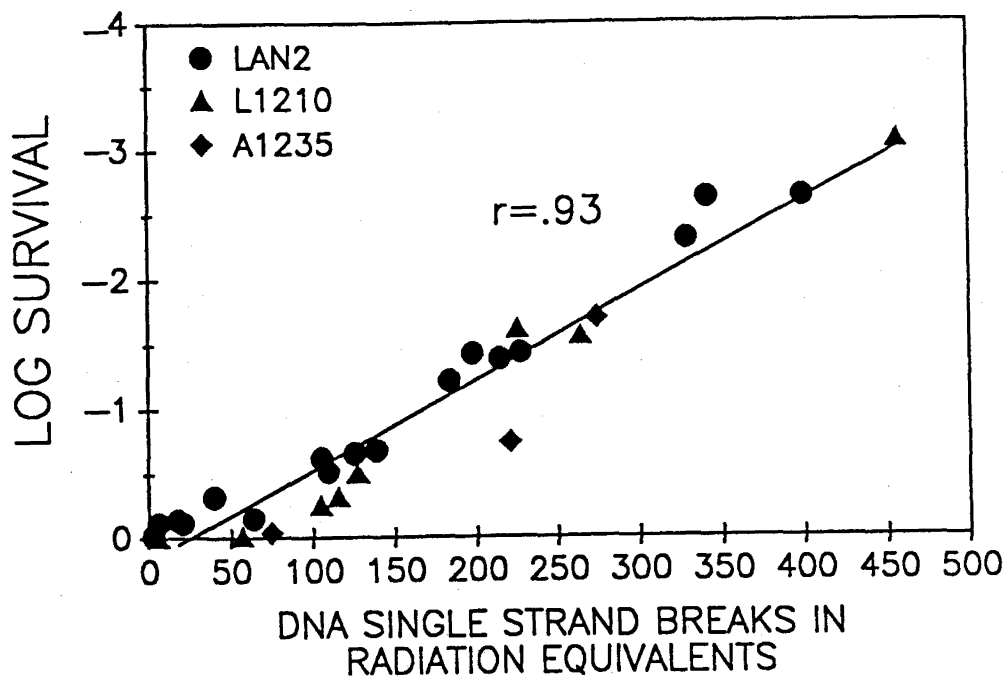
FIGURE 27. Computer generated regression analysis for all 3 cell lines in which the inverse log survival was plotted against DNA strand break frequency in radiation equivalents at equimolar concentrations of AF64A. The points represent the averages of at least 3 separate determinations and were taken from all survival and elution experiments, including Ch protection studies.

D. EFFECTS OF AF64A ON CHAT ACTIVITY

AF64A has been shown in previous studies to be an inhibitor of the ChAT enzyme (Sandberg et al., 1985). Therefore it was of interest to determine what effect AF64A had on ChAT activity in LAN2 cells. ChAT activity was measured in the LAN2 cells following exposure to AF64A. The absolute values for ChAT varied among the experiments, so data were calculated as a percent control ChAT activity. The fact that there were experiment-to-experiment variations in ChAT activity could be expected with cells grown in culture. Cholinergic tumor cell lines can vary in the amount of ChAT activity dependent on days in culture, density and differentiation of the cells (Rosenberg et al., 1971; Prasad and Vernadakis et al., 1972; Prasad and Mandal, 1973; Lucas et al. , 1979; Kirshner et al., 1986; Acheson and Rutishauser, 1988). Since experiments were performed on days when the cells might have been at different densities (not measured) this could account for differences seen in the absolute ChAT values.

There were consistent, statistically significant, decreases in ChAT levels when LAN2 were exposed to AF64A for one hour, although these decreases were not large. This is in agreement with other workers who found decreases in ChAT in cells grown in culture following exposure to AF64A (Sandberg et al., 1985). Apparently the drug has the ability to alkylate the enzyme near the active site, thereby preventing its activity (Sandberg et al., 1985). When the drug was removed after a one hour exposure to AF64A and ChAT activity was measured 24 hours later, the activity was still significantly lower than control. However by 48 hours there was an apparent rebound increase in ChAT activity, so that

it was greater than control values. This phenomenon has also been seen

with other cytostatic agents such as cytosine arabinoside (Kirshner, et

al., 1986) and X-ray and dibutyryl cAMP (Prasad and Vernadakis, 1972;

Prasad and Mandal, 1973). The authors postulated that by inhibiting

cell division, the cells go through a differentiation process in which

they extend neurites to become mature cells. When the cytostatic agent

is removed the cells do not start to divide again. In previous studies

it has been shown that FAPy derivatives are inhibitors of DNA polymerase

and block chain elongation and thus cell division (Boiteaux and Laval,

1983). Thus AF64A in LAN2 cells may cause enough damage to halt cell

division, yet does not immediately kill the cells. The cells which have

ceased dividing synthesize increasing amounts of ChAT enzyme as have

other cells exposed to cytostatic agents (Prasad and Mandel, 1973). It

is difficult to account for the fact that cells which were exposed to

cytotoxic doses of AF64A were able to regenerate the ChAT enzyme when

neuronal cells do not. However colony formation assays measure the

ability of the cell to replicate and divide, not those which may live

but were unable to divide. In previous studies, which measured cell

death using a lactate dehydrogenase assay to measure cell lysis, little

immediate cell death occurred in response to 100 uM AF64A treatment

(Sandberg et al., 1985). Thus, although cells which were exposed to

AF64A may undergo a differentiation process in which they cease to

divide and do not form colonies, they may accumulate higher levels of

enzymes per cell than cells which are not differentiated and are

continuously dividing. This would account for increased levels of ChAT

activity at concentrations of AF64A that caused tremendous decreases in

colony formation. Other studies have shown that DNA repair occurs more efficiently in transcriptionally active genes than in the genome overall in tumor cells (Bohr et al., 1985). Thus perhaps the ChAT gene is more rapidly repaired than other genes, and the cells are able to synthesize enzymes although they are not able to divide and form colonies.

CHAPTER VI

CONCLUSIONS

AF64A is more toxic to cells which are cholinergic or presumably have higher rates of choline transport, than to cells which are non-cholinergic. This is in agreement with animal studies which show that when AF64A is given in low doses, so that it is specific for HAChT, it acts specifically on cholinergic neurons and causes little non-specific damage (Hortnagl et al., 1987). The degree of AF64A-induced cytotoxicity strongly correlates with the levels of DNA damage (strand breaks). These strand breaks may either be caused by apurinic sites in the DNA or possibly by intracellular Ch depletion. For the most part, the strand breaks do not appear to be alkali labile. The cells have the ability to repair the DNA damage, although some strand breaks were evident 24 hours after removal of the drug. ChAT activity studies show that the activity of the enzyme decreased with continued exposure to AF64A. However, if cells are treated for only one hour, ChAT activity eventually recovers and even exceeds control values.

AF64A has been proposed for use as a cholinotoxin in animals as a model for Alzheimer's disease. Concerns about the use of AF64A have focused around its specificity of action and its mechanism of cholinotoxicity. The data generated in this study suggest that AF64A is more toxic to cholinergic cells than to non-cholinergic cells, and that this difference in toxicity may be related to differences in Ch transport, which is in agreement with other studies on AF64A and Ch

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transport (Pittel et al., 1987). The mechanism of AF64A's cytotoxicity may be through DNA damage, which correlates very highly with cell death. Interestingly, cells from Alzheimer's patients have been shown to be deficient in the ability to repair of DNA damage produced by alkylating agents, specifically MMS (Robison et al, 1987). Perhaps the DNA damage caused by AF64A mimics the deficiencies seen in Alzheimer's patients. Thus, AF64A appears to be a potent cholinotoxin, fairly specific for the cholinergic system, although apparently non-specific at higher doses and longer exposures, and when taken up by the cholinergic system may generate DNA damage which leads ultimately to cell death.

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APPROVAL SHEET

The thesis submitted by Diane M. Barnes has been read and approved by the following committee:

Leonard C. Erickson, Ph.D.
Professor
Departments of Medicine and Pharmacology
Loyola University Stritch School of Medicine

Israel Hanin, Ph.D.
Professor and Chairman
Department of Pharmacology
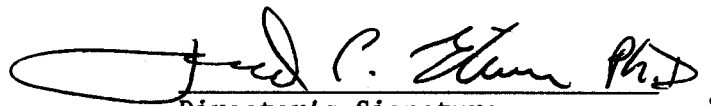Loyola University Stritch School of Medicine

Sullivan Read, Ph.D.
Assistant Professor
Department of Microbiology
Loyola University Stritch School of Medicine

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

March 27, 1989
_____
Date

_____
Director's Signature