

**p53 elevation in relation to levels and cytotoxicity of mono- and bi-functional  
melphalan DNA adducts**

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**Running title:**

p53 response to mono and bifunctional melphalan DNA adducts

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**Non-standard abbreviations used:**

PBS; Na/K phosphate, 10 mM. NaCl, 140 mM, pH 7.4.; PBST, PBS containing 0.1 % v.v Tween 20; XTT, 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate.

## Abstract

The hypothesis tested was that bifunctional DNA adducts formed by a nitrogen mustard-based anti-cancer drug were more efficient than monofunctional adducts at causing elevation of p53, consistent with the difference in cytotoxicity. Human leukaemia cell line ML-1 was exposed for 1 hour to melphalan, or its monofunctional derivative, monohydroxymelphalan. Levels of DNA-adducts, measured by specific immunoassay, were linearly related to concentration of alkylating agent.

Monohydroxymelphalan formed twice as many adducts as did equal concentrations of melphalan. After removal of alkylating agent, adduct levels were maintained or increased slightly up to 8 h and then declined by 27 – 44 % by 24 h. Alkaline elution analyses confirmed the absence of detectable DNA inter-strand cross-links in cells exposed to monohydroxymelphalan. DNA single strand breaks were detected following monohydroxymelphalan but not melphalan. Levels of p53 were quantified by sensitive fluorogenic ELISA at intervals up to 24 h after exposure of cells to various concentrations of melphalan and monohydroxymelphalan. The level of initially formed DNA adducts needed to cause elevation of p53 from a base-line level of 0.5 ng/mg total protein to 2 ng/mg were 5 to 8-fold higher for monohydroxymelphalan than melphalan. The concentrations of melphalan and monohydroxymelphalan ( $\pm$ S.D.) causing 50 % growth inhibition were  $1.2 \pm 0.4$  and  $28.1 \pm 1.6$   $\mu$ g/ml respectively, a difference of 23-fold. The adduct levels induced by these exposures were 9.3 and 420 nmoles / g DNA for melphalan and monohydroxymelphalan respectively, a difference of 45-fold which is considerably greater than the difference in efficacy at elevating p53.

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DNA damaging anti-cancer drugs and carcinogens each induce several different types of DNA modifications. It is widely accepted that the cytotoxic and anti-cancer effects of reagents such as bifunctional alkylating agents and platinum compounds result, to a significant or predominant extent, from the formation of DNA cross-links, particularly inter-strand cross-links (Ducore et al., 1982; Hansson et al., 1987; Ross et al., 1978). This is consistent with the well established fact that, for several classes of DNA-reactive drugs, two alkylating groups per molecule are necessary for cytotoxic and anti-cancer efficacy (Ross, 1962; Tokuda and Bodell, 1987; Monks et al., 2001). In addition to the direct consequences of cross-links on molecular processes, it is widely held that DNA damaging anti-cancer drugs cause cell death by initiating apoptosis. e.g. (Evans et al., 1994; Fan et al., 1994). In this context, the mechanisms by which cells detect and respond to DNA damage are important and should be similar to cytotoxicity in their dependency on bi- versus monofunctionality of an alkylating agent. An important response to DNA damage is p53 elevation, especially since, in certain cells, this has been implicated in inducing apoptosis (Lowe et al., 1993).

Inter-strand cross-links form only a small minority of the total DNA adducts formed by cross-linking drugs. In the case of nitrogen mustard compounds such as melphalan, the majority of adducts formed are monofunctional (Osborne and Lawley, 1993; Osborne et al., 1995b). Mechanisms that sense DNA damage are poorly understood (Iliakis et al., 2003) and there is a paucity of information to define the relationships between biochemical responses of cells to DNA damaging agents and quantities of specific types of DNA damage. For example, increases in p53 levels following exposure of cells to nitrogen mustard agents could be triggered by all or just certain of

the various types of DNA modification, as exemplified in an analysis of the relationships between p53 response and specific types of DNA adducts resulting from exposure to the anticancer drug Mitomycin C (Abbas et al., 2002). Attempts to fully understand cellular responses to drug exposure are further complicated by changes with time in the quality and quantity of modifications present in the DNA due to the differing kinetics of their formation and their repair. A detailed analysis of these relationships for various classes of DNA damage is relevant to understanding the mechanisms of damage detection that underlie biochemical responses and also the relationship between p53 response and drug action.

The results presented here concern the bifunctional nitrogen mustard drug melphalan (Fig. 2). Unlike Mitomycin C (Abbas et al., 2002) this agent alkylates DNA exclusively at guanine N7 and adenine N3 (Tilby et al., 1990; Osborne and Lawley, 1992; Osborne and Lawley, 1993; Osborne et al., 1995a). Previously we have described the preparation of a monofunctional derivative of melphalan, monohydroxymelphalan (Fig. 2) that is free of contamination with the bifunctional compound (Tilby et al., 1998). The DNA adducts formed by monohydroxymelphalan were compared to melphalan and shown to be identical in nature and DNA sequence-related distribution, except that, as predicted, cross-linked products (guanine-guanine and guanine-adenine) were not formed following the monohydroxymelphalan treatment (Tilby et al., 1998). We have also described a sensitive immunoassay for DNA adducts induced by melphalan (Tilby et al., 1987). This was shown to be applicable to clinical specimens (Tilby et al., 1993) and, with equal sensitivity, to adducts formed by monohydroxymelphalan (Tilby et al., 1998). These tools permit detailed comparison of the cellular effects of chemically equivalent mono- and bi-

functional adducts formed by a drug which is representative of, and relevant to, a large number of chemically and mechanistically related drugs, including agents currently being developed for targeted therapies (Melton et al., 1996).

We present here results showing quantification and characterisation of DNA damage induced in cells by melphalan and monohydroxymelphalan, rates of DNA repair and cytotoxic effects. We also describe the initial characterisation of the p53 response to this damage. The data show that, compared to adducts formed by monohydroxymelphalan, the adducts formed by melphalan are 4 to 8-fold more effective at inducing p53, but this difference is considerably less than the difference in cytotoxic efficacies.

## Materials and Methods

**Cell lines.** The human cell lines MCF7 (breast carcinoma) and Raji (leukaemia) were obtained from the American Type Culture Collection. The human myeloblastic leukaemia cell line ML-1 was obtained from the European Collection of Animal Cell Cultures (Porton, U.K.).

**Culture conditions and drug exposure.** All cells were grown in Hepes-buffered RPMI 1640 medium (Gibco/BRL, Paisley, UK) supplemented with foetal calf serum (10 % v/v), glutamine (300 mg/L), penicillin (50 U/mL), streptomycin (50 µg/mL) and neomycin (100 µg/mL) at 37 °C, 5 % CO<sub>2</sub>. Monohydroxymelphalan was prepared as described previously (Tilby et al., 1998). Solutions of melphalan (from Sigma) and monohydroxymelphalan were prepared in acidified ethanol (Tilby et al., 1993) immediately before use and then diluted into culture medium to give final ethanol concentrations of 1 % v/v for controls and for all exposures to alkylating agents. Cells were incubated with drug for 1 h at 37 °C and then drug was removed from the cells by centrifugation (190 g, 5 min, 25 °C) and washing with PBS. When cells were to be incubated further, washing was with pre-warmed medium and cells were subsequently resuspended in fresh medium at their original density.

**Cytotoxicity.** ML-1 cells ( $5 \times 10^5$  / ml) were exposed to drug for 1 h, washed by centrifugation with PBS and resuspended in medium. Aliquots of cell suspensions (100 µl) were transferred to wells of 96-well plates and incubated for 6 days before adding XTT reagent (Roehm et al., 1991) (Roche Diagnostics Ltd). OD<sub>465</sub> was measured after a further incubation (6 h). Cells (>200 per sample) were scored for

frequency of apoptotic nuclear morphology by fluorescence microscopy using cultures fixed with methanol : glacial acetic acid (3 : 1) and stained with Hoechst dye 33258 (10  $\mu$ M).

**Antibodies.** Mouse monoclonal antibodies against p53 (DO1 and PAb1801) and p21 (Ab-1) were from Oncogene Research Products. Rabbit anti-p53 polyclonal antiserum was obtained from Scottish Antibody Production Unit (Carluke, Lanarkshire, Scotland).

**Irradiation.** Cells, in culture medium, either as suspensions (ML-1) or whilst attached to 25cm<sup>2</sup> flasks (MCF7) were exposed to gamma irradiation from a <sup>137</sup>Cs source (Nordion, Gammacell 1000) at 3.64 Gray/min.

**Preparation of cell lysates for p53 analyses.** Lysis solution was 150 mM NaCl, 50 mM Tris/HCl pH8, 5 mM EDTA, 1 % NP40. Protease inhibitors (aprotinin, pepstatin, chymostatin, leupeptin - each at 1  $\mu$ g/ml, 0.5 mM benzamidine, 0.5 mM phenylmethanesulfonyl fluoride and 1 mM dithiothreitol) were added immediately before use. MCF7 cells were washed with cold PBS and 400  $\mu$ l lysis solution added per culture flask. Each flask was then placed on ice for 30 minutes before the cells were scraped and transferred to a 1.5 ml centrifuge tube. ML-1 cells were washed with PBS by centrifugation (190 g, 5 min, 25 °C). To each pellet of approximately 1x10<sup>7</sup> cells, 500  $\mu$ l of lysis solution was added, followed by incubation for 30 minutes on ice. All lysates were centrifuged (18,000 g, 30 min, 4 °C) and the supernatants collected.



**Protein estimation.** Total protein concentrations of cellular lysates were determined in relation to bovine serum albumin (BSA) standards using the BioRad DC Protein Assay (Bio-Rad Laboratories Ltd).

**SDS-PAGE electrophoresis and immunoblotting.** To cell lysates, concentrated Laemmli sample buffer was added to give the following final concentrations: Tris-HCl, pH 6.8 (62.5 mM), glycerol (10 % v/v), SDS (2 % w/v), bromophenol blue (0.005 % w/v) and 2-mercaptoethanol (5 % v/v). Samples were heated at 95 °C for 4 min and analysed using 5 % stacking and 13 % separating gels. Equal quantities (75 µg) of protein from each sample were loaded. Separated proteins were transferred to nitrocellulose membranes which were then incubated (1 h, 20 °C) in a solution of dried milk powder (5 % w/v) in PBST. Membranes were incubated with primary antibody (1 h, 20 °C), washed several times with PBST, and then incubated with a horseradish peroxidase-conjugated goat anti-mouse second antibody. After several further washes with PBST, final detection was by enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, UK).

**ELISA for p53.** A sandwich immunoassay was developed for the quantification of p53. Monoclonal anti-p53 antibody (DO-1 or 1801) solution (500 ng/ml) in NaHCO<sub>3</sub> solution (1 M, pH 9.6) was added (50 µl / well) to 96-well ELISA plates (Greiner Labortechnik, Stonehouse, U.K., medium or high bind grades). After incubation overnight at 4 °C, the plates were washed once with PBS and then incubated with blocking solution (6 % w/v BSA in PBS, 200 µl per well, 2 h at 20 °C). All subsequent washing steps were with PBST. The plates were washed (4x) and then cell lysate or p53 standard (recombinant p53 kindly provided by Prof. D.P. Lane) diluted

in sample buffer (150 mM NaCl, 50 mM Tris, 1 % w/v BSA, 0.1 % v/v NP40, 0.02 % w/v NaN<sub>3</sub>, pH 8.0) was added (50 µl per well). After incubation overnight at 4 °C, the plates were washed (4x) and 50 µl of rabbit anti-p53 antiserum diluted 20,000 fold in ELISA buffer (PBS containing 1 % w/v BSA and 0.1 % Tween 20) was added to each well. After incubation (2 h, 20 °C) and then further washes (4x), goat anti-rabbit biotin conjugate (Sigma, diluted x10,000 in ELISA buffer) was added (50 µl/well) and the plates incubated for 1 hour (37 °C). The plates were then washed (4x) and streptavidin β-galactosidase conjugate added (Roche, 50 µl/well, diluted x 5,000 in ELISA buffer containing 10 mM MgCl<sub>2</sub>). After incubation (1 h, 37 °C), the plates were washed (5x), and substrate solution was added (50 µl per well of 80 µg/ml 4-methylumbelliferyl β-D-galactoside, in PBS containing 10 mM MgCl<sub>2</sub>). After incubation (37 °C, 3 h) the fluorescence was measured in a plate reader (Dynex, MFX, excitation 354 nm, emission 445 nm).

*Validation of p53 ELISA:* Standard curves were linear and reproducible (inter-assay CV for slope of the standard curve = 30 %). Inter-assay CV for determination of p53 content of a quality control standard (lysate of RAJI cells) was 14 % and the average detection limit (concentration of p53 giving a signal 2 S.D. above the signal for zero p53) was 0.05 ng p53/ml. Fig. 1 shows the good agreement between ELISA and immunoblot assays performed on the same lysates of MCF-7 cells at various times after exposure to ionising radiation (4 Gy). The changes are consistent with previous data for this cell line (Wieler et al., 2003).

**DNA extraction and immunoassay of DNA adducts.** DNA extraction and competitive ELISA methods using monoclonal antibody MP5/73 were performed as described elsewhere (Tilby et al., 1987; Tilby et al., 1991; Tilby et al., 1993). This technique shows equal sensitivity for DNA adducts formed by melphalan and monohydroxymelphalan, (Tilby et al., 1998) nevertheless, independent standards of DNA alkylated with the appropriate radioactively labelled alkylating agent were included in every assay.

**Alkaline elution.** This assay was performed essentially according to Kohn et al (1981). ML-1 cells were incubated for 24 h in medium containing  $^{14}\text{C}$ -thymidine (specific activity = 52 mCi/mmol, 0.016  $\mu\text{Ci/ml}$ ) or, for internal standards,  $^3\text{H}$ -thymidine (specific activity = 41 mCi/mmol, 0.1  $\mu\text{Ci/ml}$ ). Before use, all cells were incubated for a further 4 h in the absence of radiolabelled thymidine. Internal standard cells were irradiated with 3 Gy ionising radiation. All cells were kept at 0 °C until lysis. Equal numbers ( $10^6$ ) of experimental and standard cells were mixed and collected on a polycarbonate filter (Whatman, 0.2  $\mu\text{m}$  pore size, in the dark at 4 °C). Following lysis (2 ml of 2 % w/v SDS, 25 mM EDTA, pH 9.7) filters were incubated with a further 1.5 ml of lysis buffer containing proteinase K (0.5 mg/ml, 1 h at 20 °C). During elution of DNA (pH 12.1, 33  $\mu\text{l/min}$ ) 8 x 90 min fractions were collected per filter and DNA elution was calculated from radioactivity, measured using a liquid scintillation counter.

## Results

**ELISA analysis of formation and removal of DNA adducts.** ML-1 cells were exposed for 1 h to a range of concentrations of melphalan or monohydroxymelphalan and then, without further incubation, DNA was extracted and adduct levels determined by competitive ELISA based on antibody MP5/73 which recognises adducts formed by melphalan and monohydroxymelphalan on guanine N7 (Tilby et al., 1987; Tilby et al., 1993; Tilby et al., 1998). Over the ranges used, the levels of adducts were linearly related to concentration of alkylating agent (Fig. 2). The mean ( $\pm$  S.D.) slope of the linear regression lines from 3 independent experiments were 8.2 ( $\pm$  1.5) and 17.3 ( $\pm$  5.6) (nmoles adduct) / (g DNA) per  $\mu$ g alkylating agent /ml, for melphalan and monohydroxymelphalan, respectively. Monohydroxymelphalan induced the formation of 2.1-fold higher levels of immunoreactive adducts than did the same concentrations of melphalan. To investigate the change in adduct level with time, ML-1 cells were exposed for 1 h to concentrations of melphalan or monohydroxymelphalan that gave similar initial adduct levels (melphalan at 10 or 20  $\mu$ g/ml; monohydroxymelphalan at 5 and 10  $\mu$ g/ml). After further incubation for various periods in drug-free medium, samples were harvested and frozen. Adduct-levels (Fig. 3) were maintained or increased slightly (by 10 – 46 %) during the first 8 h after removal of alkylating agent and declined by 27 – 44 % of the peak levels by 24 h.

**Alkaline elution analyses.** Biologically, the most significant difference between melphalan and monohydroxymelphalan is probably the inability of the latter compound to form DNA inter-strand cross-links. This inability was confirmed by alkaline elution experiments using ML-1 cells exposed for 1 h to various

concentrations of melphalan or monohydroxymelphalan. For analysis of cross-links, the cells were irradiated prior to lysis. Other samples were analysed without irradiation both to act as controls for the cross-linking assays and in order to investigate the possibility that DNA single strand breaks were formed during repair of melphalan and monohydroxymelphalan adducts. Initial assays were performed immediately after the exposure period (Fig. 4). As expected (Ducore et al., 1982; Hansson et al., 1987; Millar et al., 1986; Ross et al., 1978), melphalan caused a concentration-dependent reduction in the elution rate of DNA from irradiated cells but did not affect the elution of DNA from non-irradiated cells. Monohydroxymelphalan did not cause a significant alteration to the elution rate of DNA from irradiated cells, consistent with the expected lack of inter-strand cross-links. However, monohydroxymelphalan did cause a concentration dependent increase in the elution of DNA from non-irradiated cells, indicating that exposure to monohydroxymelphalan leads to the formation of DNA strand-breaks. The changes with time in levels of cross-links and strand breaks for melphalan and monohydroxymelphalan, respectively, were studied in order to determine repair rates and to relate damage levels at different times to p53 induction. ML-1 cells were exposed to melphalan (10  $\mu\text{g/ml}$ ) or monohydroxymelphalan (5  $\mu\text{g/ml}$ ) for 1 h. These concentrations were chosen so as to result in similar initial levels of total adducts (Fig. 2) and to be as low as possible, consistent with reliable quantification of DNA damage. Cells exposed to melphalan were irradiated before analysis. For these cells, the retention of DNA increased with time and then decreased (Fig. 5). Cells exposed to monohydroxymelphalan were not irradiated and, at all time points studied, the DNA from these cells eluted to a significantly greater extent than did DNA from control

cells (Fig. 5). This degree of elution did not change markedly over the time period studied.

**Elevation of p53.** ML-1 cells were exposed to melphalan or monohydroxymelphalan at a range of concentrations for 1 h and were then washed free of drug. Untreated cells were subjected to the same washing steps. After further incubation for 0, 1, 3, 5 and 24 h, samples of cells were harvested and stored at  $-20^{\circ}\text{C}$ . As positive controls, in each experiment additional cells were exposed to 4 Gy of ionising radiation and were harvested after further incubation for the same time intervals. The ELISA method involving antibody DO-1 was used to measure the p53 concentrations in relation to total protein in cell lysates. Very similar data was obtained when antibody 1801 was used instead of DO-1. The samples used for measurement of DNA adducts (Fig. 2) were removed from the same cultures as were used for the p53 assays. Following ionising radiation, in each of 3 separate experiments, the level of p53 increased by about 5-fold after 3 h and had started to decline by 5 h (Fig. 6). Untreated cells consistently showed a small variation in p53 level following the washing step. Exposure of ML-1 cells to monohydroxymelphalan resulted in higher adduct levels than exposure to equal concentrations of melphalan (Figs. 2 and 3). Since p53 response is induced by DNA damage, it is most relevant to present the data in relation to adduct level. Figs. 7 and 8 show averaged data from the three independent experiments. Fig. 7 shows the relationship between adduct level (immediately after exposure) and p53 level at various times after exposure to melphalan or monohydroxymelphalan. Adducts formed by melphalan were more effective than adducts formed by monohydroxymelphalan at inducing an increase in p53 at the 3, 5 and 24 h time points. This difference is shown more clearly when selected data are

plotted against time. Data for exposure to melphalan and monohydroxymelphalan at 5 and 10  $\mu\text{g/ml}$  or 10 and 20  $\mu\text{g/ml}$ , respectively, are compared in Fig. 8. These pairs of concentrations each induced similar initial levels of DNA adducts. The p53 elevation following exposure to melphalan or monohydroxymelphalan followed similar time courses, but the p53 levels induced by melphalan attained levels 2 to 3 fold higher than were induced by equal levels of adducts formed by monohydroxymelphalan.

**Elevation of p21.** ML-1 cells were exposed to melphalan (20  $\mu\text{g/ml}$ ) or monohydroxymelphalan (10  $\mu\text{g/ml}$ ) for 1 h. These concentrations induced similar levels of total DNA adducts (Figs. 2 and 3). At various times after the end of the treatment, samples were removed and stored at  $-20\text{ }^{\circ}\text{C}$ . Parallel cultures were irradiated (4 Gy) or untreated. Cells were lysed and analysed by immunoblotting for p21 expression (Fig. 9). Clear increases in p21 level were detectable at 3 and 5 h after ionising radiation. There was a strong signal for p21 at 24 h after melphalan with a weak signal at 5 h. Weak signals were seen at 24 h after monohydroxymelphalan.

**Assessment of cytotoxicity.** Cytotoxicity induced by melphalan and monohydroxymelphalan was assessed by growth inhibition assay using the XTT method (Fig. 10A). The  $\text{IC}_{50}$  value for monohydroxymelphalan ( $28.1 \pm \text{S.D. } 1.6\text{ }\mu\text{g/ml}$ ) was approximately 23 times higher than for melphalan ( $1.2 \pm 0.4\text{ }\mu\text{g/ml}$ ). DNA adduct levels formed at those  $\text{IC}_{50}$  values indicate that adducts formed by melphalan were approximately 45 times more toxic than the adducts formed by monohydroxymelphalan (Table 1). Observations on nuclear morphology (Fig 10B), trypan blue exclusion and total cell numbers (data not shown) indicated that, after exposure to concentrations of melphalan or monohydroxymelphalan (even up to 50

μg melphalan/ml), there was no overt sign of cells dying at up to 24 h post-exposure.

Frequency of apoptotic cells became elevated only at later time points.



## Discussion

The 2.1-fold higher efficiency of DNA-adduct formation by monohydroxymelphalan compared to melphalan (Fig. 2), despite its lower molar alkylating capacity, was unexpected and illustrates the importance of monitoring adduct levels. It cannot be attributed to an assay artefact (Tilby et al., 1998) or an influence of local DNA sequence on adduct recognition by antibody MP5/73 (McCartney et al., 2001), especially since melphalan and monohydroxymelphalan displayed indistinguishable patterns of sequence-dependent alkylation (Tilby et al., 1998). The higher adduct levels after exposure to monohydroxymelphalan did not result from slower adduct removal (Fig. 3) but were probably due to differences in cellular uptake or inactivation processes. melphalan is taken up by amino-acid transport systems which discriminate between melphalan and hydrolysed melphalan (Begleiter et al., 1979).

The transient increase in levels of immunoreactive adducts following removal of alkylating agent (Fig. 3) resembled previous observations (Tilby et al., 1993) and is attributed to continued reaction of retained intracellular melphalan. Reduction in adduct levels with time did not result from dilution through DNA synthesis or from selective loss of highly damaged cells because, during 24 h following exposure to melphalan or monohydroxymelphalan there were no significant increases in intact or apoptotic cells and adduct levels in individual cells were relatively homogeneous (Frank and Tilby, 2003). Spontaneous hydrolytic loss of melphalan-guanine adducts was probably only a minor contribution since the reaction half life was 110 h at pH7 (Osborne and Lawley, 1993). The melphalan concentrations used (10 and 20  $\mu\text{g/ml}$ ) were many fold higher than its  $\text{IC}_{50}$  (1.2  $\mu\text{g/ml}$ ). However, it is unlikely that adduct

removal was significantly diminished through toxicity because similar rates of adduct loss were seen with comparable levels of monohydroxymelphalan adducts. These had been induced by concentrations of monohydroxymelphalan (5 and 10  $\mu\text{g/ml}$ ) much lower than its  $\text{IC}_{50}$  (28  $\mu\text{g/ml}$ ). The low rates of removal of melphalan-DNA adducts were consistent with data on clinical samples and a different cell line (Tilby et al., 1993) and with the inefficient removal of melphalan-DNA adducts by mammalian 3-methyladenine-DNA glycosylase (Mattes et al., 1996). Importantly, throughout the period over which DNA inter-strand cross-links, single strand breaks and p53 levels were studied, immunoreactive DNA adducts remained at relatively high levels.

Bifunctional DNA adducts were undetectable in purified DNA reacted with monohydroxymelphalan (Tilby et al., 1998). The present work confirms the absence of detectable DNA inter-strand cross-links in cells exposed to monohydroxymelphalan even at 50  $\mu\text{g/ml}$  (Fig. 4). The increased rate of elution of DNA from cells exposed to monohydroxymelphalan and not irradiated (Fig. 4), indicated the formation of DNA strand breaks. The approximately steady-state level of strand breaks over 2-24 h following exposure to monohydroxymelphalan (Fig. 5) presumably resulted from ongoing DNA repair.

Cells exposed to melphalan probably carried single strand breaks at similar levels to those formed following exposure to monohydroxymelphalan. Failure to detect single strand breaks in non-irradiated cells exposed to melphalan (Fig. 4) illustrates masking of single strand breaks by the simultaneous presence of inter-strand cross-links. Retardation of DNA elution following melphalan exposure reached a maximum several hours after removal of the drug (Fig. 5), consistent with other reports (Ross et

al., 1978) which concluded that inter-strand cross-links form through slow second-arm reactions. An alternative explanation, apparently not ruled out, was that the reduction in elution rate with time resulted from a reduction in single strand breaks following completion of repair of the more numerous monofunctional adducts. The present data excludes this explanation for melphalan in ML-1 cells. However, the delayed increase in inter-strand cross-links could have resulted from delayed increase in total alkylation rather than just second-arm reactions.

The observation that melphalan was about 23 fold more cytotoxic than monohydroxymelphalan is consistent with data for other alkylating agents *in vivo* (Ross, 1962) and in cell lines (Tokuda and Bodell, 1987; Monks et al., 2002; Palom et al., 2002). DNA adducts induced by melphalan were actually about 45 times more cytotoxic than the adducts induced by monohydroxymelphalan.

ML-1 cells are wild-type for p53, exhibit normal p53 responses, express low levels of p53 (comparable to normal tissues and many tumours) and have been used for key studies of p53 (Kastan et al., 1991; Houser et al., 2001; Abbas et al., 2002). The low levels of p53 observed in this study and the extent and time-scale of changes induced by ionising irradiation (Fig. 6) are consistent with previous data (Kastan et al., 1991). For equivalent overall levels of DNA adducts, melphalan was more effective than monohydroxymelphalan at inducing p53 elevation (Fig. 7). The levels of DNA adducts necessary to cause elevation of p53 by about 4-fold (to 2 ng/mg protein) at various times after exposure to alkylating agent were estimated (Table 1). Adducts formed by melphalan were 5 to 8 fold more effective at causing this elevation than adducts formed by monohydroxymelphalan. This did not result from greater

persistence of total melphalan adducts and was therefore due to intra- and/or inter-strand DNA cross-links. These could have triggered p53 elevation directly (Achanta et al., 2001; Unsal-Kacmaz et al., 2002) or through repair intermediates such as DNA double-strand breaks (Huang et al., 1996; Nelson and Kastan 1994).

Level of DNA adducts is an important determinant of biochemical response to drug exposure and a good basis for comparing experimental and clinical conditions. However, relevant data on adduct levels in patients is available for very few drugs. Levels of DNA adducts formed in ML-1 cells following 1 h exposures to 2.5 and 5 µg/ml melphalan were in the same range as levels present in normal peripheral blood mononuclear cells removed from patients 1 h after administration of high dose melphalan (Tilby et al., 1993). In a plasma cell leukaemia patient, adduct levels in tumour cells were higher at about 80 nmoles / g DNA, equivalent to the levels in ML-1 cells following 1 h exposure to 10 µg/ml melphalan. Thus the p53 responses described here were induced by levels of DNA damage shown to be clinically relevant.

The present study is unusual in combining quantification of both p53 and specific DNA modifications. However, the data do not exclude the possibility that differential post-translational modification of p53 following exposure to melphalan and monohydroxymelphalan leads to markedly different down-stream consequences (e.g. Gottifredi et al., 2001; Meyer et al., 1999). From semi-quantitative assessment, p21 expression (Fig. 9) appeared to follow a similar pattern to overall p53 elevation and thus provided no evidence for differential effects on this aspect of p53 function.

In the present work, elevation of p53 has been related to overall growth inhibition rather than specifically apoptosis. The role of apoptosis in response to p53 activation will depend on the cell-line specific expression of many down-stream components (Villunger et al 2004). Loss of p53 function has been linked to drug resistance through failure to engage apoptosis (Lowe et al., 1993). If, as implied in this model, elevation of p53 plays a significant role in mediating the cytotoxic effects of melphalan then, after clinically relevant drug exposures, p53 responses should be induced much more effectively by melphalan than monohydroxymelphalan adducts. Melphalan-DNA adducts were 8-fold more efficient at causing p53 elevation than monohydroxymelphalan adducts (Table 1). However, this was considerably less than the 45-fold higher efficiency of melphalan adducts at causing cytotoxicity. Thus, either initial p53 elevation was of minor importance for melphalan-induced cytotoxicity or different patterns of post-translational modifications of p53 were induced by the two agents.

At its  $IC_{50}$  concentration (1.2  $\mu$ g /ml or 9.3 nmoles adducts / g DNA) melphalan would not cause significant elevation of p53 (Fig. 7). This contrasts with monohydroxymelphalan which, at its much higher  $IC_{50}$  concentration (28.1  $\mu$ g /ml or 420 nmoles adducts / g DNA) caused a very marked elevation of p53 (Fig. 7). In the absence of more detailed analysis of p53 quality, the current data is consistent with a model in which melphalan and related drugs are effective anti-cancer agents because they form cytotoxic cross-links at levels of overall DNA damage too low to trigger a major p53 response to the initial damage. Thus, cell cycle progression would commence with a critically damaged genome. In contrast, initial p53 response could

be more important for the cytotoxic effects of monohydroxymelphalan where higher levels of DNA adducts are necessary to kill cells.

It will now be of interest to define in greater detail the comparative effects of melphalan and monohydroxymelphalan on various p53 post-translational modifications and on down-stream consequences, such as changes in the expression of the numerous p53 dependent genes (Villunger et al 2004). Comparison of effects of melphalan and monohydroxymelphalan on cell cycle progression and various DNA damage responses such as formation of nuclear foci of phosphorylated histone H2AX are also being undertaken. The present analysis of DNA damage formed by matched mono and bi-functional alkylating agents constitutes a foundation on which such further studies of cell responses can be based.

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We thank D.R. Newell and B.T. Golding for valuable discussions, B.W. Durkacz for guidance with the alkaline elution and valuable comments on the manuscript, H. McCartney and S. Kyle for assistance with certain techniques and D.P. Lane for providing recombinant p53 protein.

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**Footnotes**

**Footnote to title:**

This work was supported by Cancer Research UK and the UK Leukaemia Research Fund.

## Figure legends

**Fig. 1.** Comparison of results from p53 ELISA and immunoblot analyses for MCF7 cells exposed to ionising radiation. Cells were exposed to zero ( $\Delta$ ) or 4 Gy ( $\blacktriangle$ ) and were lysed immediately or after the indicated period of incubation. Each point on the graphs represents mean p53 level ( $\pm$  S.E.) of 3 separate ELISA determinations. These were performed on the same lysates as were used for the immunoblot analyses shown above the graphs.

**Fig. 2.** Relationship between concentration of alkylating agent and level of DNA adducts in ML-1 cells immediately after a 1 h exposure to melphalan ( $\blacktriangle$ ) or monohydroxymelphalan ( $\circ$ ). DNA adducts were assayed by competitive ELISA using antibody MP5/73. Typical data from one of 3 separate experiments. Each point represents mean of 3 replicate ELISA determinations. Error bars represent SEM where this is greater than the symbol.

**Fig. 3.** Change in levels of DNA adducts with time after the end of a 1 h exposure of ML-1 cells to melphalan at 10 ( $\bullet$ ) or 20 ( $\blacktriangle$ )  $\mu$ g/ml or to monohydroxymelphalan at 5 ( $\nabla$ ) or 10 ( $\circ$ )  $\mu$ g/ml. Adduct levels were determined by competitive ELISA using monoclonal antibody MP5/73. Each point represents the mean ( $\pm$  S.E.) of three separate experiments, in each of which the ELISA assays were performed in triplicate.

**Fig. 4.** Alkaline elution analysis. The relationship between concentration of alkylating agent and the proportion of DNA retained on filters at 20 % retention of  $^3\text{H}$ -labelled internal standard DNA. ML-1 cells were labelled with  $^{14}\text{C}$ -thymidine and exposed for 1 h to melphalan ( $\Delta$ ) or monohydroxymelphalan ( $\bullet$ ). Each point represents the mean result of three separate experiments  $\pm$  SEM, where this is greater than the symbol. Before lysis, the cells were either irradiated (4 Gy, panel A) or not irradiated (B). All experimental samples were mixed with an internal standard of ML-1 cells that had been labelled with  $^3\text{H}$ -thymidine and irradiated (3 Gy).

**Fig. 5.** Alkaline elution analysis. Relationship between time of incubation post-exposure to alkylating agent and the proportion of DNA retained on filters at 20 % retention of  $^3\text{H}$ -labelled internal standard DNA. ML-1 cells labelled with  $^{14}\text{C}$ -thymidine were exposed for 1 h to either 10  $\mu\text{g/ml}$  melphalan ( $\blacktriangle$ ) or 5  $\mu\text{g/ml}$  monohydroxymelphalan ( $\circ$ ). After further incubation, cells were harvested for analysis. Cells exposed to MEL were irradiated (4 Gy). Each point represents the mean result of three separate experiments  $\pm$  SEM where this is greater than the symbol. Other conditions were as for Fig. 4.

**Fig. 6.** Change in p53 levels after exposure of ML-1 cells to ionising irradiation (4 Gy,  $\blacktriangle$ ), melphalan (10  $\mu\text{g/ml}$ ,  $\bullet$ ), monohydroxymelphalan (10  $\mu\text{g/ml}$ ,  $\circ$ ) or no damaging agent ( $\Delta$ , dotted line). Levels of p53 were determined by ELISA using antibody DO-1. Each point represents mean ( $\pm$  SEM) of three separate ELISA determinations. Typical data from one of three separate experiments.



**Fig. 7.** Levels of p53 after exposure to melphalan (●) or monohydroxymelphalan (○). Cells were harvested 0, 3, 5 and 24 h (panels A, B, C, D respectively) after the end of a 1 h exposure to alkylating agent. Each point represents the mean of 3 separate experiments in each of which the p53 levels were determined in triplicate by ELISA using antibody DO-1.

**Fig. 8.** Changes in levels of p53 with time after the end of a 1 h exposure to melphalan (●) or monohydroxymelphalan (○). Upper panel: Cells exposed to melphalan at 10 µg/ml and monohydroxymelphalan at 5 µg/ml (mean initial adduct levels ± SEM were 87 ±7 and 75 ±5 nmoles /g DNA respectively). Lower panel: Cells exposed to melphalan at 20 µg/ml and monohydroxymelphalan at 10 µg/ml (mean initial adduct levels ± SEM were 165 ±19 and 175 ±21 nmoles /g DNA respectively). Each point represents the mean of 3 separate experiments in each of which the p53 levels were determined in triplicate using an ELISA based on antibody DO-1.

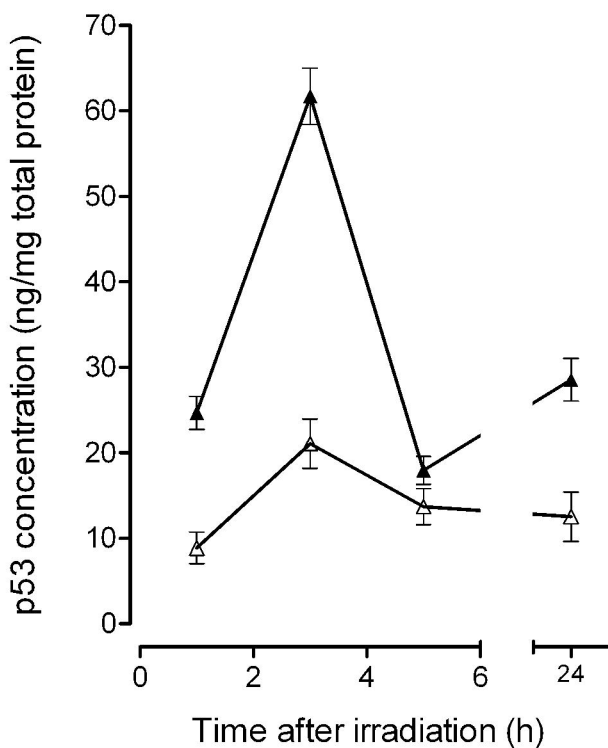
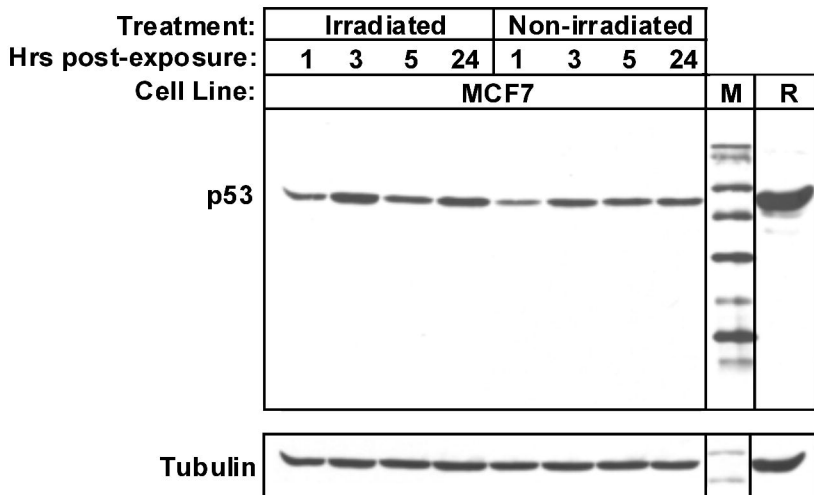
**Fig. 9.** Immunoblot analysis of p21 protein in ML-1 cells following exposure to DNA damaging agents. Exponentially growing ML-1 cells were treated with 4 Gray ionising radiation, 10 µg/ml monohydroxymelphalan, 20 µg/ml melphalan or mock-treated with drug diluent. Cells were harvested at 1, 3, 5 and 24 hours post-treatment. Aliquots of cell lysates containing 75 µg of protein were loaded and analysed using antibody, Ab-1.

**Fig. 10.** Comparison of the effects of melphalan and monohydroxymelphalan on ML-1 cells. A: Growth inhibition assays using the XTT method. Melphalan (●), monohydroxymelphalan (▲); B: frequency of apoptotic cells determined from morphology of Hoechst dye-stained nuclei. No treatment (●); melphalan at 5 (▲) and 50 (▼) µg/ml; monohydroxymelphalan at at 5 (▲) and 50 (▼) µg/ml.

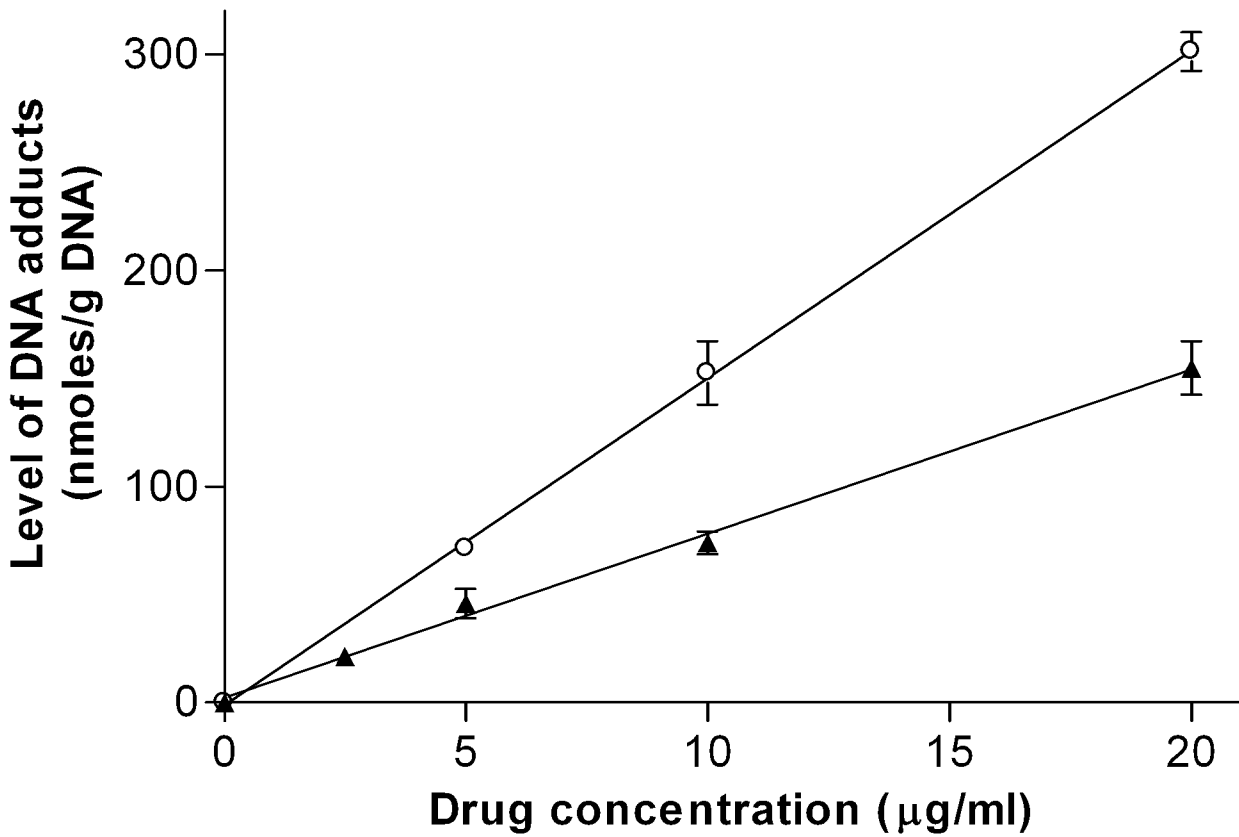
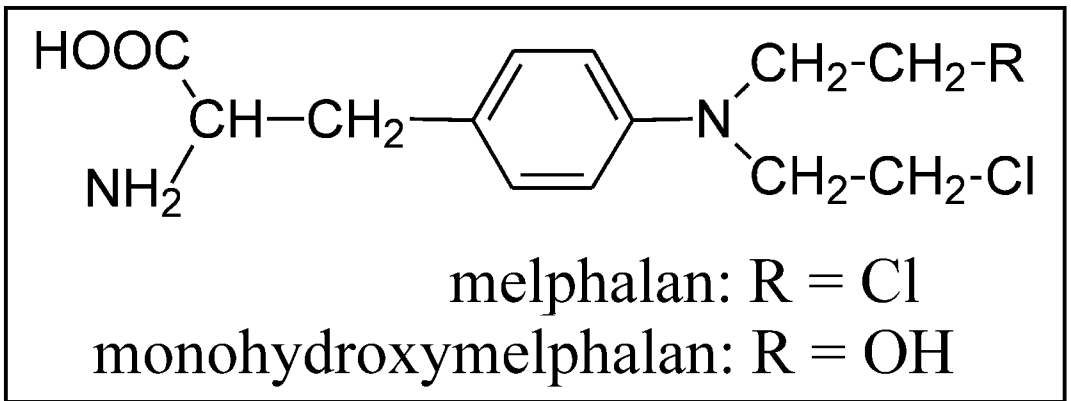
**Table 1.** Levels of DNA adducts necessary to cause p53 level to increase to 2 ng/mg protein at various times after exposure to melphalan or monohydroxymelphalan for 1 h.

End point	Time after exposure	Adduct level (nmole/g DNA)		Ratio of adduct levels. <u>monohydroxymelphalan</u> melphalan
		melphalan	monohydroxy-melphalan	
P53 level = 2 ng/ml	3 h	72	322	4.5
	5 h	31	238	7.7
		24 h	32	243
50 % growth inhibition <sup>a</sup>	6 days	9.3	420	45

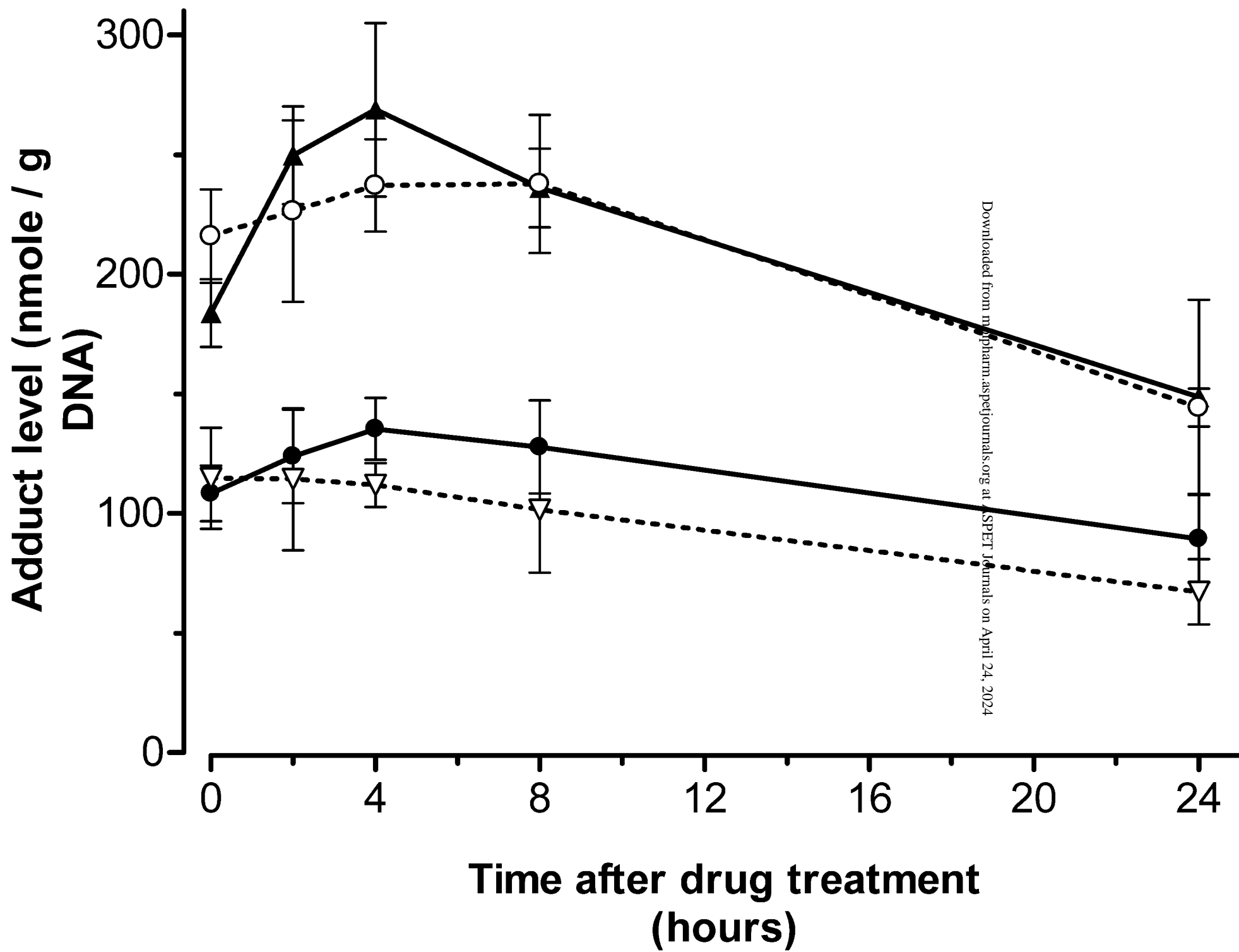
<sup>a</sup> 50% growth inhibitory concentrations ( $\pm$  S.D.) were 1.2 ( $\pm$  0.4) and 28.1 ( $\pm$  1.6)  $\mu$ g/ml for melphalan and monohydroxymelphalan respectively. Adduct levels induced by these exposures were calculated from linear regression lines fitted to the combined data from 3 sets of data for DNA adduct levels determined in relation to concentration of melphalan or monohydroxymelphalan, as stated in the text.



**Fig. 1**



**Fig. 2**



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Fig. 3

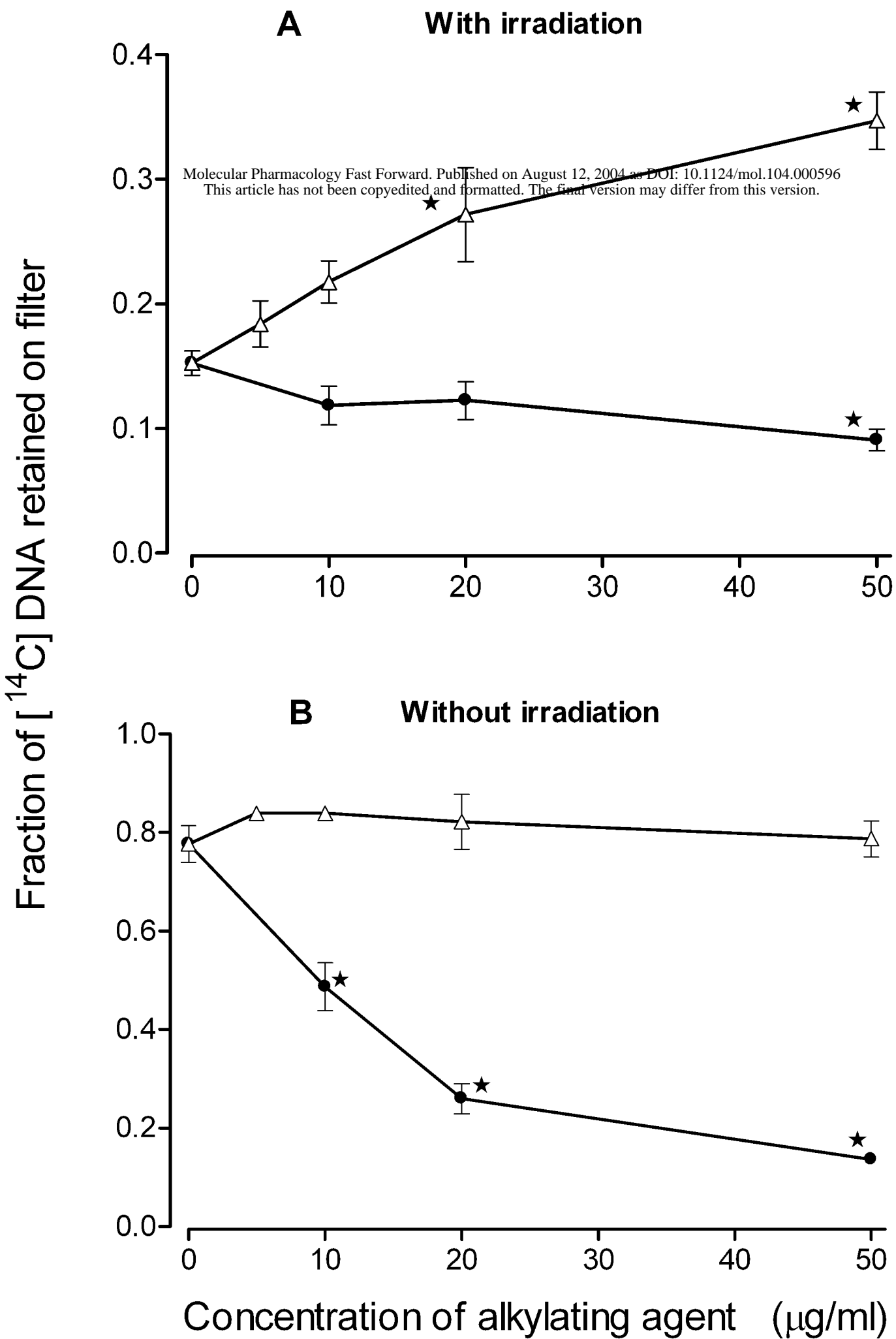


Fig. 4

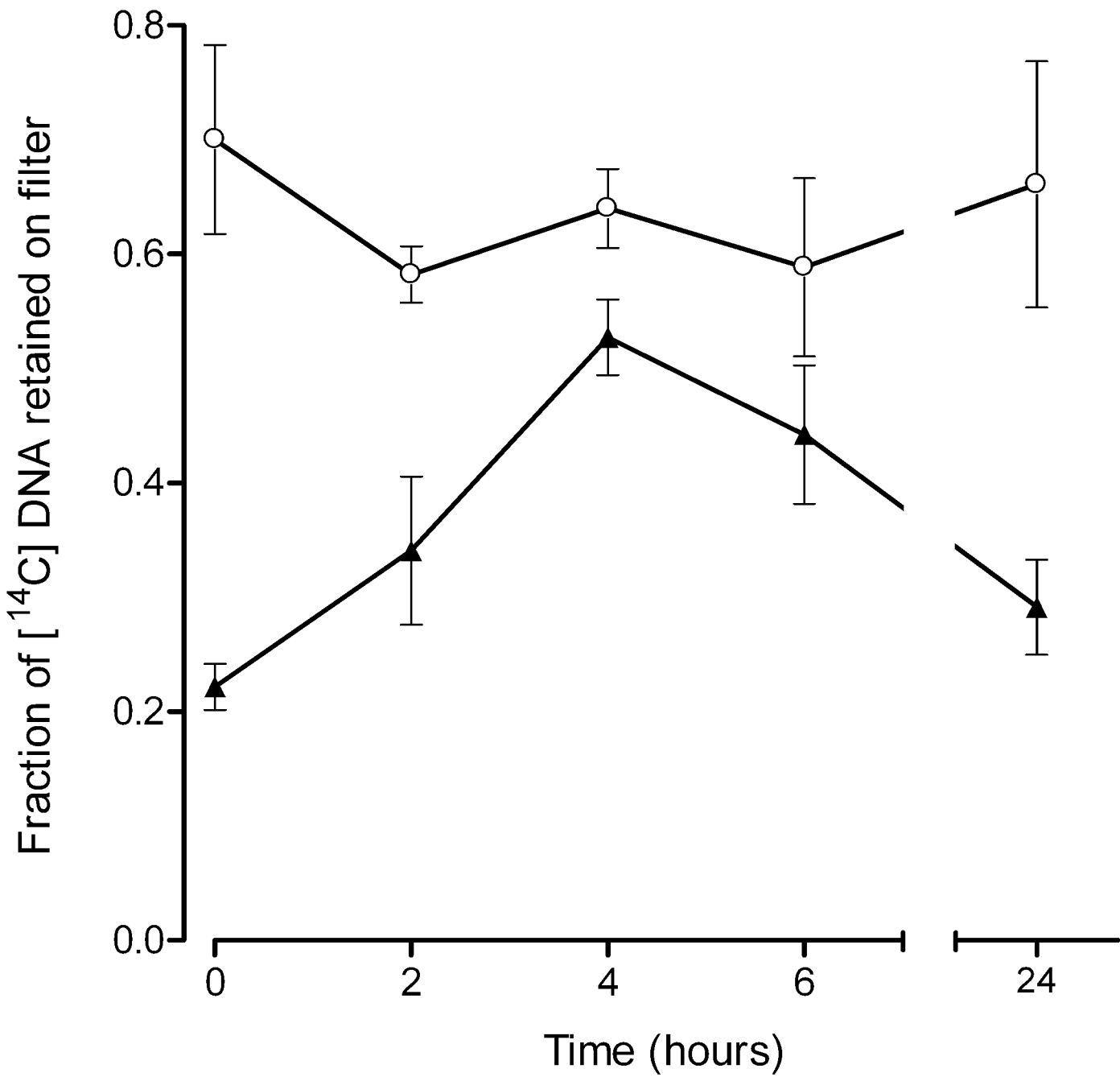


Fig. 5



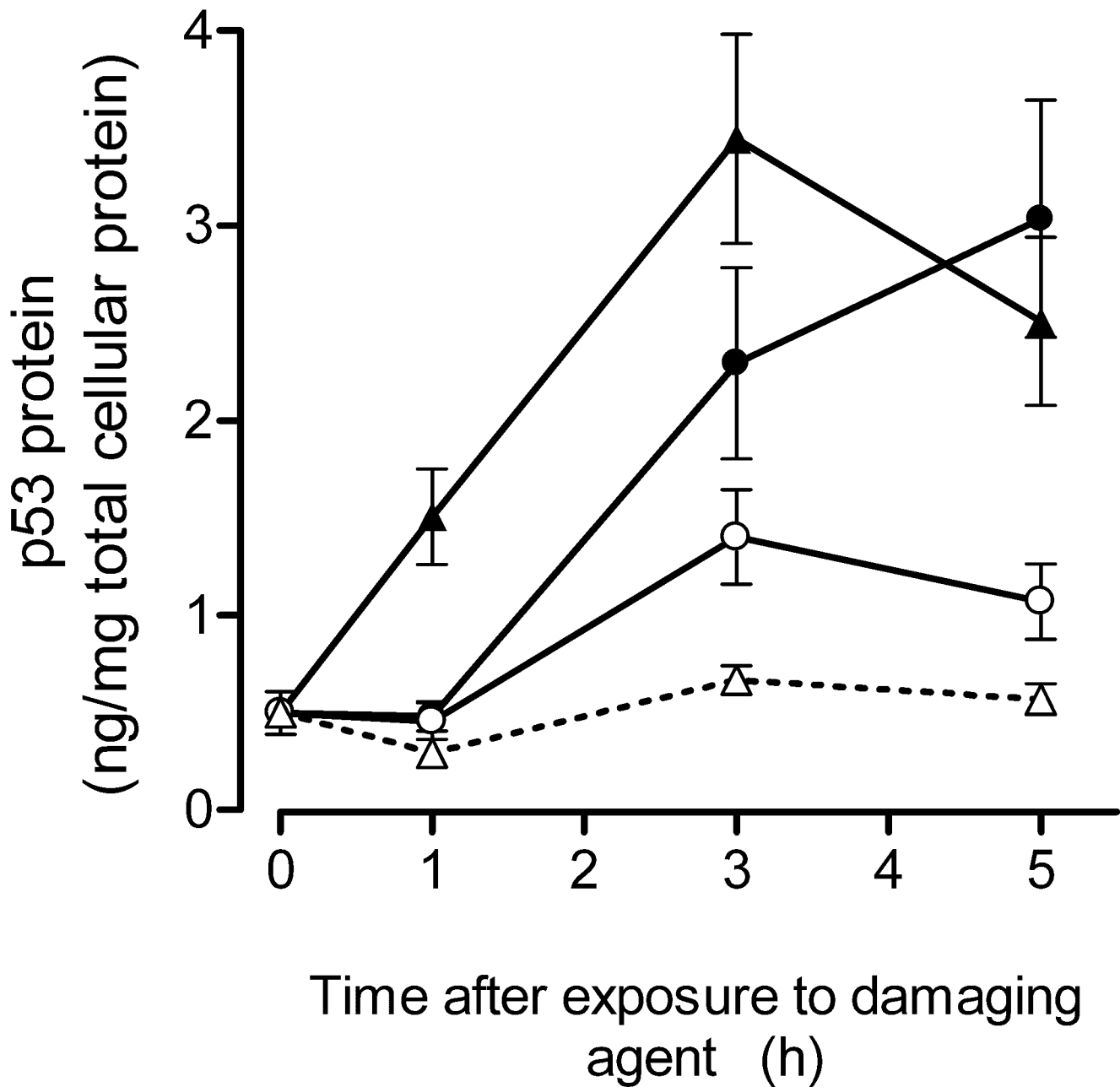
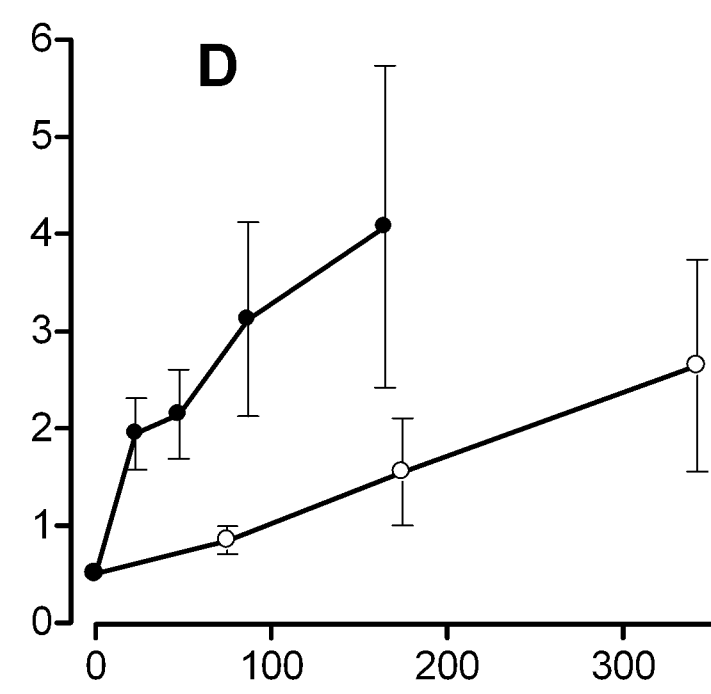
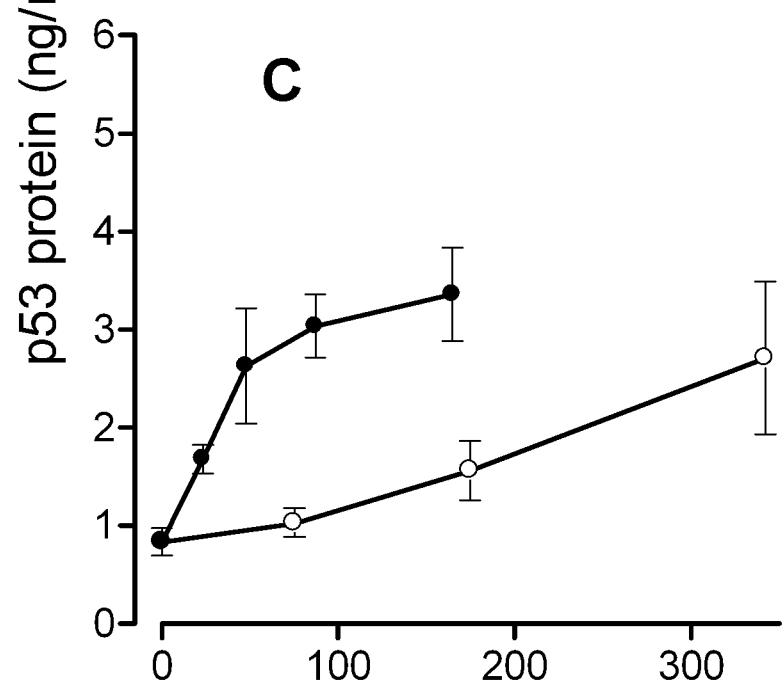
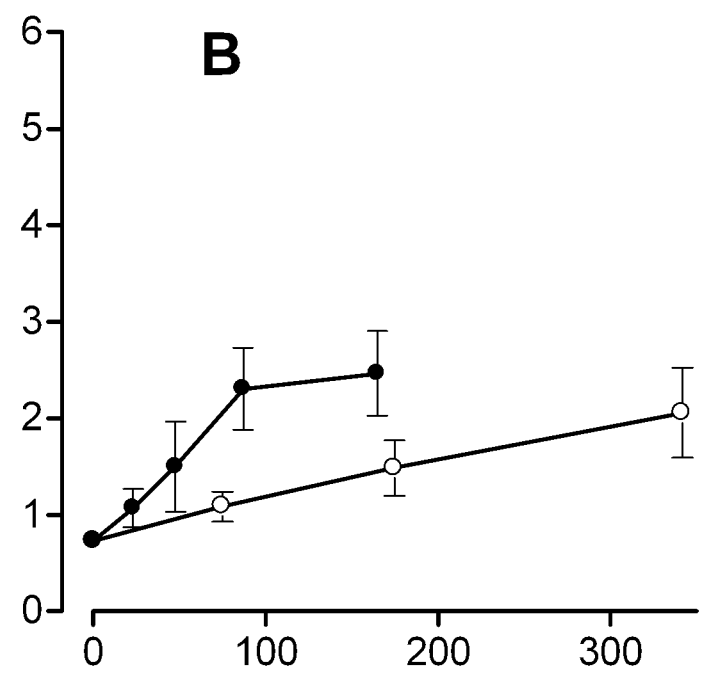
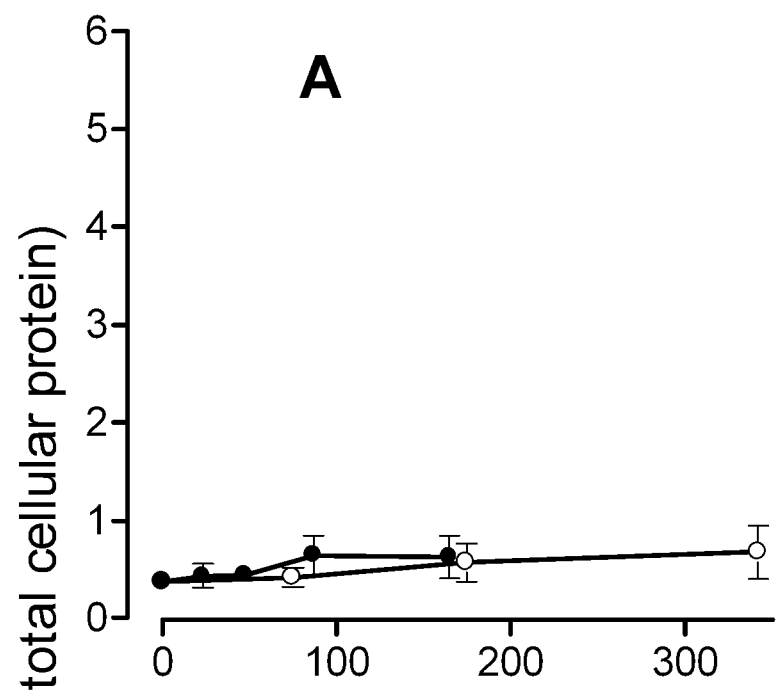


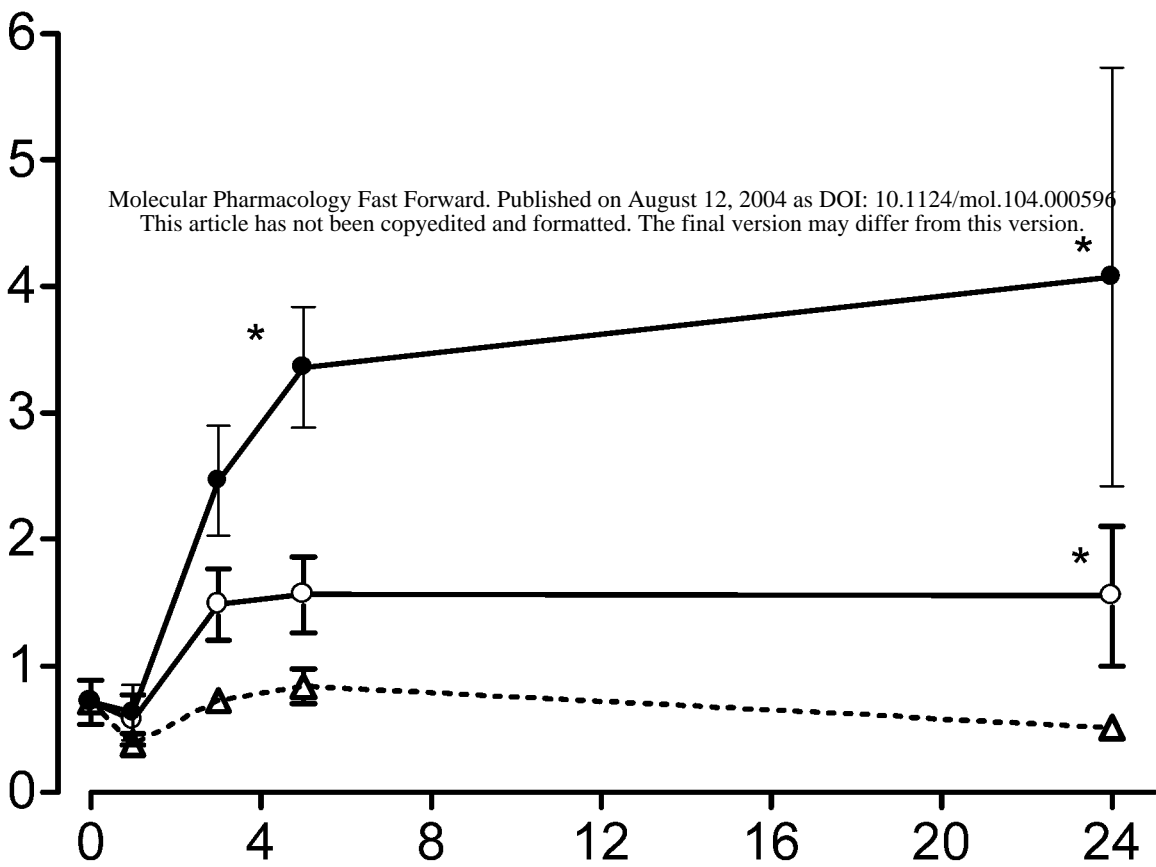
Fig. 6



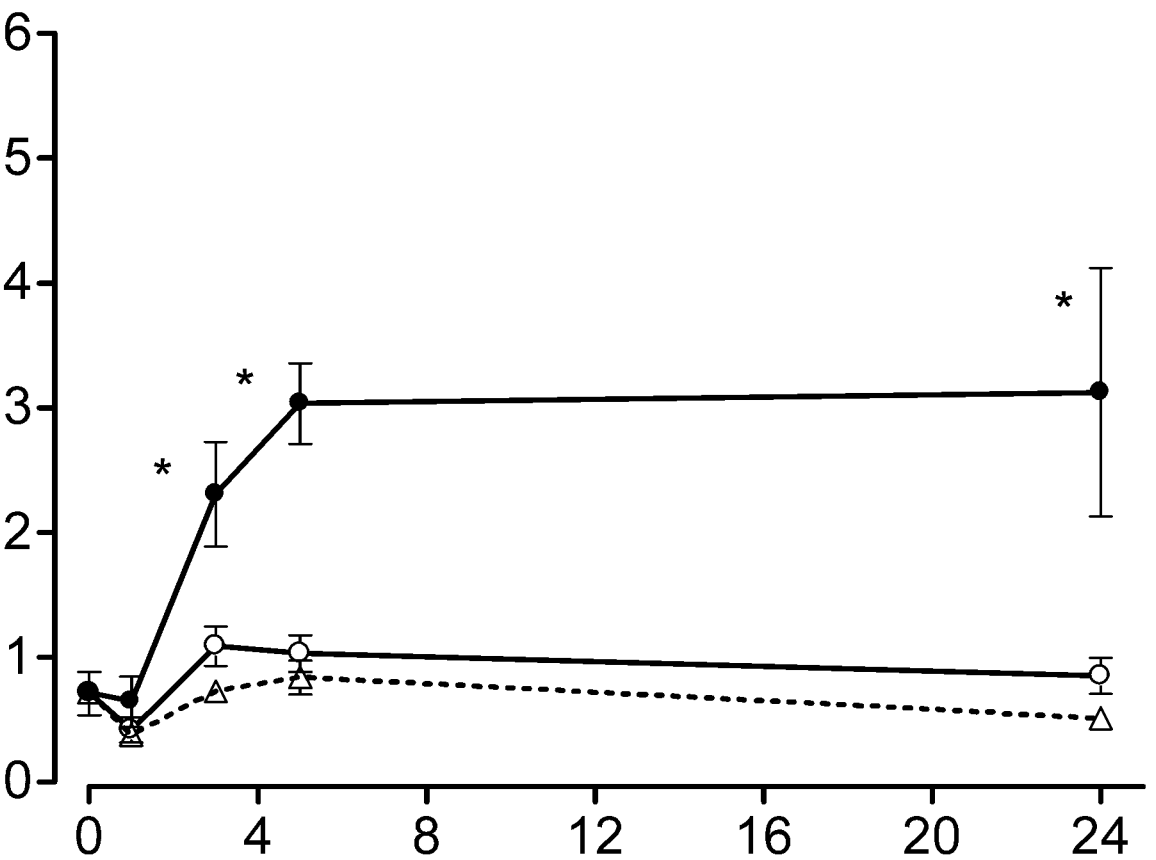
Level of DNA adducts (nmoles/g DNA)

Fig. 7

**p53 protein (ng/ml of total protein)**



**p53 protein (ng/ml of total protein)**



**Time after exposure to alkylating agent (h)**

Fig. 8

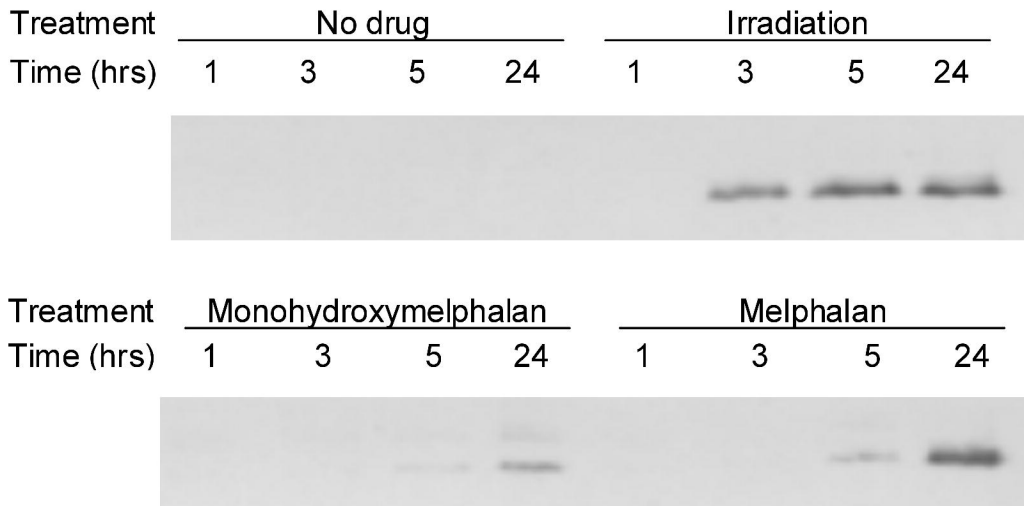
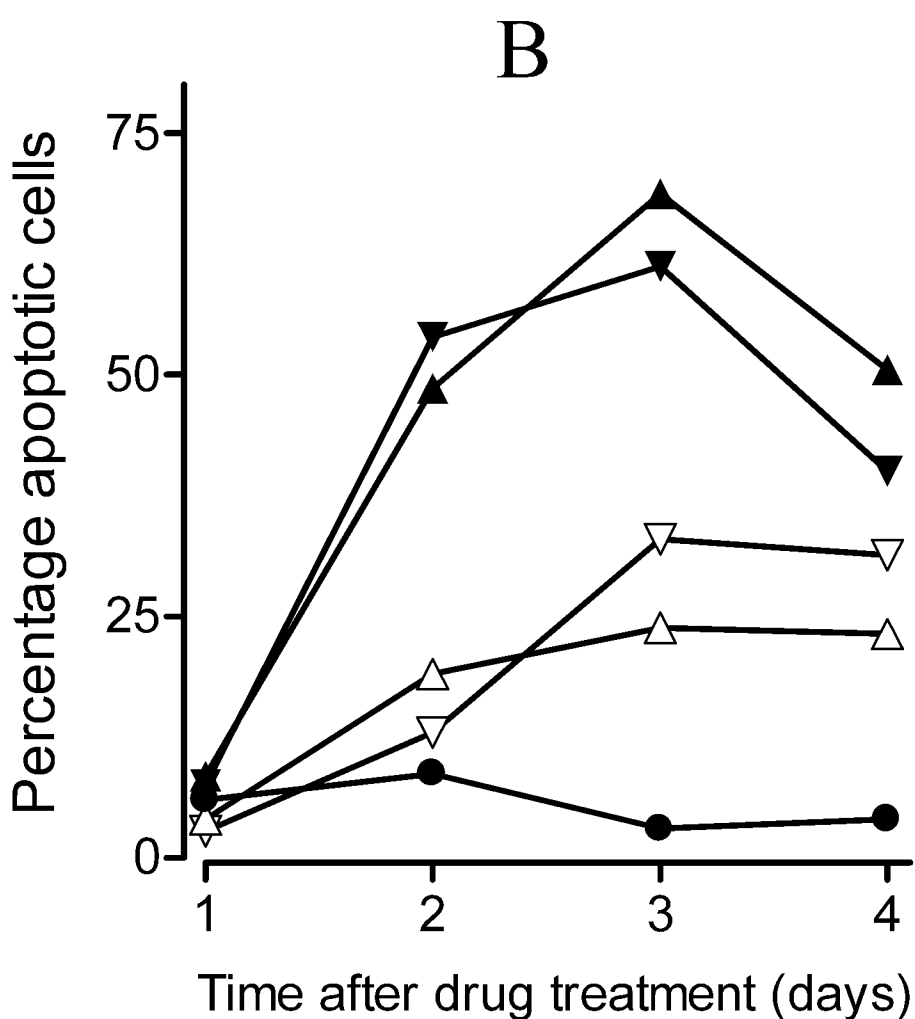
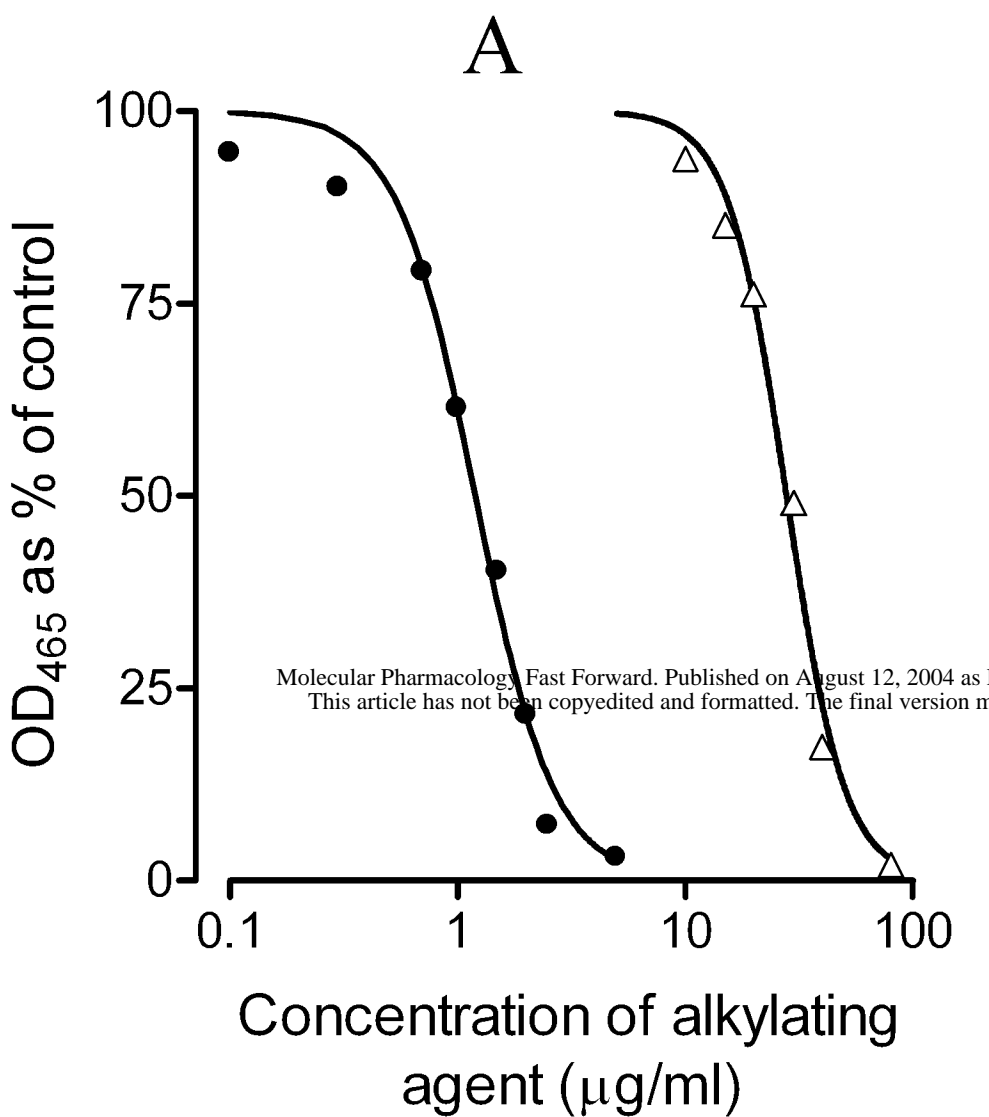


Fig. 9



**Fig. 10**