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Cellular Topoisomerase I Inhibition and Antiproliferative Activity by MJ-III-65 (NSC 706744), an Indenoisoquinoline Topoisomerase I Poison

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Running title: Cellular Topoisomerase I inhibition by MJ-III-65

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ABBREVIATIONS: CPT, camptothecin; Top1, topoisomerase I; NSC 314622, 5,6-dihydro-5,11-diketo-2,3-dimethoxy-6-methyl-8,9-methylenedioxy-11*H*-indeno (1,2-*c*)isoquinoline; MJ-III-65, 6-[3-(2-Hydroxyethyl)amino-1-propyl]-5,6-dihydro-2,3-dimethoxy-8,9-methylenedioxy-5,11-dioxo-11*H*-indeno[1,2-*c*]isoquinoline; DPC, DNA-protein crosslinks; SSB, Single-strand breaks; ICE, Immuno complex of enzyme.

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Abstract

To overcome camptothecin's (CPT) lactone instability, reversibility of the drug-target interaction and drug resistance, attempts to synthesize compounds that are CPT-like in their specificity and potency yet display a unique profile have been underway. In this pursuit, we have identified one of the idenoisoquinoline derivatives (MJ-III-65; NSC 706744) with both similarities and differences from CPT. MJ-III-65 traps topoisomerase I (Top1) reversibly like CPT but with different DNA sequence preferences. Consistent with Top1 poisoning, protein-linked DNA breaks were detected in cells treated with MJ-III-65 at nanomolar concentrations. These MJ-III-65-induced protein-linked DNA breaks were resistant to reversal even after an hour of drug removal as compared to CPT, which completely reversed. Studies in human cells in culture found MJ-III-65 to be cytotoxic. Furthermore, limited cross-resistance was observed in camptothecin-resistant cell lines. MJ-III-65 also exhibits antitumor activity in mouse tumor xenografts.

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Introduction

Since DNA topoisomerase I (Top1) has been identified as a cancer therapeutic target, designing potent Top1 inhibitors has been actively pursued. Thus far, camptothecin (CPT) derivatives are the only Top1 inhibitors approved for clinical use by the FDA (Vanhoefer et al., 2001; Zunino and Pratesi, 2004). However, chemical instability, rapid cleavage complex reversibility after drug removal, drug resistance and side effects compromise the efficacy of CPT derivatives. Consequently, there is a need for additional therapeutic agents that while being CPT-like in their specificity and potency would induce novel DNA cleavage patterns, have extended durations of action and reduced toxicity profiles.

To discover, design and develop novel Top1 inhibitors, a COMPARE analysis was carried out using CPT as a seed. As a result, we identified NSC 314622 (Fig. 1) as having a similar tumor cell growth inhibitory profile as CPT in the NCI cytotoxicity screen. Further analysis showed NSC 314622 to be a Top1 inhibitor (Kohlhagen et al., 1998). Though not as potent as CPT, its chemical stability, unique Top1 cleavage sequence preference and slower cleavage complex reversibility made NSC 314622 a good lead compound. By chemically modifying NSC 314622, we synthesized indenoisoquinoline derivatives to increase Top1 inhibition and cancer cell cytotoxicity (Cushman et al., 2000; Fox et al., 2003; Strumberg et al., 1999). As reported earlier (Antony et al., 2003), one of the derivatives MJ-III-65 (NSC 706744), with an amino alcohol instead of a methyl at the N-6 position of the parent compound (Fig. 1), is a potent inhibitor

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of Top1. MJ-III-65 preferentially traps Top1 at sites with cytosine (C) immediately 5' from the Top1 cleavage site as compared to a thymine (T) for CPT. Moreover, MJ-III-65 remains active against Top1 enzymes that are resistant to CPTs, homocamptothecins and indolocarbazoles (Antony et al., 2003).

Having demonstrated MJ-III-65 to be a Top1 inhibitor comparable to CPT in its potency *in vitro* (Antony et al., 2003; Cushman et al., 2000), this study was carried out to ascertain MJ-III-65's *in vivo* potential. Here we show that in human leukemic cells MJ-III-65 causes DNA-protein crosslinks and Top1 cleavage complexes that are markedly more resistant to reversal after drug removal than those produced by CPT. Also, CPT-resistant cells remained sensitive to MJ-III-65 and MJ-III-65 exhibits antitumor activity in mouse tumor xenografts.

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Materials and Methods

Drugs, Enzymes, and Chemicals. Camptothecin was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). The synthesis of MJ-III-65 has been described previously (Cushman et al., 2000). Etoposide (VP-16) was purchased from Sigma (St. Louis, MO). Drug stock solutions were made in dimethylsulfoxide at 100 mM for VP-16, 10 mM for CPT and 5 mM for MJ-III-65. Aliquots were stored at –20^{o}C and further dilutions were made in dimethylsulfoxide immediately before use. The final concentration of dimethylsulfoxide in the reactions did not exceed 10% (v/v).

Recombinant human Top1 (Top1) was purified from TN5 insect cells (HighFive, Invitrogen Corp., San Diego, CA) using a baculovirus construct for the N terminus truncated human Top1 cDNA as described previously (Zhelkovsky and Moore, 1994). The C21 human Top1 monoclonal antibody was a generous gift from Yung-Chi Cheng, Yale University and human Top2 antibodies were purchased from TopoGEN (Columbus, OH). DNA polymerase I (Klenow fragment), dNTP [where N is A (adenosine), C (cytosine), G (guanosine) or T (thymine)], agarose and polyacrylamide/bis were purchased from GIBCO BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). DNA quick spin columns were purchased from Roche Diagnostics Corporation (Indianapolis, IN). [α-^{32}P]-deoxyGTP was purchased from DuPont-New England Nuclear (Boston, MA).

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Top1 reactions. The 161 base pair fragment from pBluescript SK (-) phagemid DNA (Stratagene, La Jolla, CA) was cleaved with restriction endonucleases *Pvu*II and *Hind*III (New England Biolabs, Beverly, MA) in supplied NE buffer 2 (50-µl reactions) for 1 hr at 37^{o}C. Reaction products were separated by electrophoresis in a 1% agarose gel made in 1 X Tris/borate/EDTA buffer. The 161 base pair fragment was eluted from the gel slice using the QIAEX II kit (QIAGEN Inc., Valencia, CA). The pSK fragment was singly 3'-end-labeled by a fill-in reaction. Briefly, linearized pSK (200 ng) was incubated with [α^{32}P]-dGTP in 1 X labeling buffer (0.5 mM each dATP, dCTP, dTTP in 50 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$, 50 mM NaCl) in the presence of 0.5 units of the klenow fragment of DNA polymerase I. Labeled DNA was purified using mini quick spin DNA columns (Roche Diagnostics Corporation, Indianapolis, IN).

For Top1 cleavage assays, labeled DNA (~50 fmole/reaction) was incubated with 5 ng of recombinant Top1 with or without drug at 25^{o}C in 10 µl reaction buffer (10 mM Tris-Cl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 µg/ml BSA, final concentrations). For reversal experiments, the sodium dodecyl sulfate (0.5 %) stop was preceded by the addition of NaCl to a final concentration of 0.35 M followed by incubation for the indicated times at 25^{o}C.

Samples were denatured by the addition of 3.3 volumes of Maxam Gilbert loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, pH 8.0). Aliquots were separated in 16% denaturing polyacrylamide gels (7M urea) in 1 X

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Tris/borate/EDTA (89 mM Tris-borate, 2 mM EDTA pH 8.0) for 2 h at 40 V/cm at 50°C.

Imaging and quantitation were performed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

DNA-protein crosslinks (DPC) and single-strand breaks (SSB). Alkaline elution was performed to assess DNA damage by detecting DNA-protein and DNA-DNA cross-links as previously described (Bertrand and Pommier, 1995; Kohn, 1996; Kohn et al., 1981; Pommier et al., 1995). Before alkaline elution and drug treatments, human leukemic CEM cells were radiolabeled with 0.02-µCi/ml of [^{3}H]thymidine for 1 to 2 doubling times at 37^{o}C and then chased in nonradioactive medium overnight. Cells were treated with appropriate concentrations of MJ-III-65 or CPT for 1 hr. After drug treatments, cells were scraped in Hanks' balanced salt solution. For reversal experiments, the cells were cultured in drug-free medium for the appropriate time before scraping. After alkaline elution, filters were incubated at 65^{o}C with 1N HCl for 45 min and then 0.04 M NaCl were added for an additional 45 min. Radioactivity in all fractions was measured with a liquid scintillation analyzer (Packard Instruments, Meriden, CT).

DPC were analyzed under nondeproteinizing, DNA-denaturing conditions using protein-adsorbing filters (polyvinylchloride-acrylic copolymer filters, 0.8 µm pore size; Gelman Science, Ann Harbor, MI) and LS10 lysis solution (2M NaCl, 0.2% Sarkosyl, and 0.04 M disodium EDTA, pH 10). All cell suspensions were

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irradiated with 30 Gy. The DNA was eluted from filters with tetrapropylammonium hydroxide-EDTA, pH 12.1, without sodium dodecyl sulfate at a flow rate of ~0.035 ml/min. Fractions were collected at 3 h intervals for 15 h. DPC frequencies were calculated according to the bound to one terminus model formula (Ross et al., 1979) which is represented as

$$p_{cD} = (1/(1-r) - 1/(1-r_o))\, p_{bR}$$

where p_{cD} is the frequency of drug-induced DNA-protein crosslinks, p_{bR} is the frequency of X-ray-induced single-strand breaks (3000 when results are expressed in Rad-equivalents and 30 Gy is used prior to elution), and r and r_o are the fractions of the DNA eluting in the slow component in the presence and absence of drug respectively.

DNA single-strand breaks (SSB) were assessed by alkaline elution under deproteinizing, DNA denaturing conditions. Briefly, after treatment, radiolabeled cells were harvested at 4°C, loaded onto polycarbonate filters (2 µm pore size; Poretics, Livermore, CA) and lysed with sodium dodecyl sulfate buffer (0.1 M glycine, 0.025 M EDTA, 2% w/v sodium dodecyl sulfate, and 0.5 mg/ml proteinase K, pH 10). The lysis solution was washed from filters with 0.02 M EDTA, pH 10, and the DNA was eluted with tetrapropylammonium hydroxide-EDTA, pH 12.1, containing 0.1% sodium dodecyl sulfate at a flow rate of 0.035 ml/min into five fractions at 3-h intervals.

Detection of covalent Top1-DNA complexes in CEM-cells. Top1-DNA adducts were isolated using the ICE bioassay (Pourquier et al., 2000; Shaw et

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al., 1975; Subramanian et al., 1995). Briefly, 10^6 cells treated or untreated CEM cells were pelleted and immediately lysed in 1 % sarkosyl. After homogenization with a Dounce homogenizer, cell lysates were gently layered on CsCl step gradients and centrifuged at 165,000g for 20 h at 20^{o}C. Half-milliliter fractions were collected, diluted with an equal volume of 25 mM sodium phosphate buffer (pH 6.5) and applied to Immobilon-P membranes (Millipore) by using a slot-blot vacuum manifold as described previously (Pourquier et al., 2000). Top1-DNA complexes were detected using the C21 Top1 monoclonal antibody and Top2-DNA complexes using Top2 antibody using standard Western procedures.

Cell Lines and cytotoxicity assays. The human T-lymphoblastoid leukemia CEM cell line was purchased from American Type Culture Collection (Rockville, MD). The CEM/C2 cells were established as described previously (Fujimori et al., 1995; Fujimori et al., 1996). P388 and P388/CPT45 mouse leukemia cells were a kind gift from Michael R. Mattern and Randal K. Johnson (Glaxo SmithKline, King of Prussia, PA). P388/CPT-45 resistant cells were obtained by exposing CPT-5 cells (Mattern et al., 1991; Mattern et al., 1993) to stepwise increasing concentrations of CPT until they grew in the presence of 45 μM of CPT. CEM and P388 cells were maintained in RPMI 1640 (GIBCO BRL, Gaithersburg MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 2 mM L-glutamine in a 5% CO_2 incubator at 37^{o}C.

Cytotoxicity of MJ-III-65 in CEM, CEM/C2, P388 and P388/CPT45 cells was measured using the MTT (Sigma Chemical Co.) colorimetric assay

performed in 96-well plates. Cells were seeded (6000 cells for CEM and P388 and 30,000 cells for CEM/C2 and P388/CPT45) into each well with 180 µl of RPMI 1640 containing 10% FBS. Twenty µl of MJ-III-65 or CPT at each concentration were added to the wells, and incubations were continued for 3 days, after which 10 µl of MTT (5 mg/ml in PBS) was added to each well. After an additional 4-h incubation, the resulting formazan was dissolved in 100 µl of 2-propanol containing 0.04 N HCl. Optical densities were read immediately at 570 nm using a Micro Plate Reader. Determinations for all experiments were made in duplicates, and the results were expressed as mean ± SD. Percent growth was calculated relative to control (untreated cells) after 3 days of culture with control taken as 100.

Antitumor activity and toxicity of NSC-314622 and MJ-III-65 in nude mice bearing human A253 and FaDu head & neck xenografts. Athymic nude mice (nu/nu, female, 20-25 g, 8-12 weeks old) from HSD were transplanted with A253 and FaDu human head and neck xenografts. Briefly, 50 mg of non-necrotic tumor pieces was transplanted s.c. to nude mice. Both the A253 and FaDu tumors were transplanted in the same animals at different sides (right vs left). Treatment was initiated 6-8 days later when tumor weight reached 200-250 mg. Five mice per treatment group were included in all experiments. The animals were treated with either NSC 314622 (5 or 10 mg/kg/week) or MJ-III-65 (10, 25 or 50 mg/kg/week) administered i.v. push via tail vein once a week for four consecutive weeks. Both drugs were dissolved in dimethylsulfoxide and diluted to 10% dimethylsulfoxide

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solution. The two axes (mm) of tumor (L, longest axis; W, shortest axis) were measured with the aid of a Vernier caliper. Tumor weight (mg) was estimated as Tumor weight = 1/2 ($L \times W^2$). Tumor measurements were taken daily for the first 10 days and at least three times a week the first 3 weeks of post-therapy and once a week thereafter. Estimates were made of the maximum tolerated dose (MTD), defined as the maximum dose that does not cause drug-related lethality in mice and maximum weight loss < 20%, the antitumor activity (MTGI), defined as the relative tumor volume of the treated animal over control, and the tumor doubling time (TD), defined as the mean time for the tumor to reach twice the initial size.

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RESULTS

MJ-III-65 induces Top1-mediated DNA cleavage complexes with a different pattern from CPT. Induction of DNA cleavage in the presence of Top1 was tested in the PvuII/HindIII fragment of pBluescript SK(-) phagemid DNA (pSK) (Fig. 2A). The DNA cleavages produced by the indenoisoqinolines were different from the pattern observed with CPT. The lined wedge to the right of the Fig. 2A marks a CPT cleavage site that is not observed with the indenoisoquinolines. In contrast, the two open wedges show indenoisoquinoline DNA cleavage sites that are not seen with CPT. The solid wedge indicates common cleavage sites that are observed with both CPT and the indenoisoquinolines. The bands corresponding to the indenoisoquinoline-stabilized DNA cleavage sites varied in intensity between the indenoisoquinolines. Previous studies have shown the lead indenoisoquinoline NSC 314622 to be a Top1 inhibitor though not as potent as CPT. However, MJ-III-65 (Fig. 2A) exhibits Top1 inhibition comparable to CPT with differential DNA cleavage preferences for sites 44 and 62 as compared to sites 37, 97 and 119 for CPT.

Reversibility of Top1-DNA cleavage complexes induced by MJ-III-65. To compare the stability of the Top1-DNA cleavage complexes induced by CPT and MJ-III-65, salt-reversal experiments were carried out (Tanizawa et al., 1995). In pSK DNA (Fig. 2B) salt-reversal of Top1-DNA cleavage complexes was slower for MJ-III-65 (sites 44 and 62) than for CPT (sites 70 and 119). Site 92, which is

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targeted by both drugs, showed similar reversal. This reversibility of Top1-DNA cleavage complexes is consistent with the reversible trapping of Top1 cleavage complexes by MJ-III-65 (Antony et al., 2003).

MJ-III-65 induces Top1-DNA complexes in cells. The ICE (Immuno complex of enzyme) bioassay can detect topoisomerase-DNA covalent complexes in tissue culture cells or *in vivo* samples (Shaw et al., 1975; Subramanian et al., 1995). We used this assay to evaluate whether Top1-DNA complexes were detectable in MJ-III-65-treated cells. Exponentially growing CEM cells were treated with 1, 10 or 100 µM MJ-III-65, 1 µM CPT or 100 µM VP-16 for 1 hr and processed in the ICE bioassay. Fractionation of the CsCl gradient showed DNA band in fractions 7-10 and immunoblotting revealed the presence of Top1 signals in these DNA fractions for the MJ-III-65- and CPT-treated cells but not in the untreated or VP-16-treated cells (Fig. 3). Immunoblotting against Top2 was positive in the DNA fractions of the VP-16-treated cells, but not in the CPT- or MJ-III-65-treated cells. These data indicate that MJ-III-65 produces Top1- but not Top2-DNA cleavage complexes in cells and demonstrate that Top1 is a cellular target for MJ-III-65.

DNA-protein crosslinks (DPC) induced by MJ-III-65 in cells persists after drug removal. To quantitate the Top1-DNA complexes in drug treated cells, alkaline elutions were carried out to detect DNA-protein crosslinks (DPC) (Covey et al., 1989; Kohn and Pommier, 2000; Kohn et al., 2000; Pommier et al., 1994a;

Pommier et al., 1994b). Figure 4 shows that MJ-III-65 produced DPC in a concentration-dependent manner. DPC were detectable after 1 hr exposure to concentrations as low as 30 nM. The induction of DPC increased almost linearly as a function of MJ-III-65 concentration up to 1 μM. At higher concentrations, a saturation of the DPC was observed. Because Top1 cleavages are characterized by an equivalent frequency of DNA single-strand breaks (SSB) and DPC (Covey et al., 1989; Kohn, 1996; Kohn and Pommier, 2000), we also measured MJ-III-65-induced SSB by alkaline elution. Table 1 shows a near equivalence between SSB and DPC, which is consistent with Top1 inhibition by MJ-III-65.

We then studied the reversal of the DPC induced by MJ-III-65. MJ-III-65 was compared with CPT. Figure 5 shows that MJ-III-65-induced DPC were not reversible within 1 h after drug removal. By contrast, CPT-induced DNA crosslinks reversed completely within 30 min of drug removal (Covey et al., 1989; Tanizawa et al., 1994). Thus, MJ-III-65-induced DNA crosslinks persist following drug removal.

Sensitivity of CPT-resistant cell lines to MJ-III-65. Because MJ-III-65 had been previously shown to be effective in trapping CPT-resistant and mutant Top1 enzymes in DNA cleavage assays (Antony et al., 2003), we evaluated the sensitivity of human leukemia cells CEM/C2 (having the mutation N722S in Top1 and silencing of the normal Top1 allele) (Fujimori et al., 1996) to both MJ-III-65 and CPT. We used an additional cell line P388/CPT45. This cell line is cultured with 45 μM CPT and is highly resistant to CPT (Urasaki et al., 2001; Urasaki et

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al., 2000). Western blot analysis using monoclonal antibody against Top1 does not detect Top1 in P388/CPT45. The antiproliferative activity of MJ-III-65 and CPT was evaluated by MTT assays. Limited cross-resistance was seen in the CEM/C2 cell line (Fig. 6). The Top1-deficient cells (P388/CPT45 only) showed considerable resistance to MJ-III-65 at low drug concentrations (<1 μM) but not at higher doses while resistance to CPT was >2000-fold (Fig. 7). These results demonstrate that MJ-III-65-mediated cell cytotoxicity is Top1-dependent at low concentrations, and that MJ-III-65 can overcome CPT resistance resulting from Top1 mutations.

Antitumor activity and toxicity of MJ-III-65 and NSC-314622 in nude mice bearing A253 and FaDu human tumor xenografts. The data in Table 2 and Figures 8 and 9 are a summary of the antitumor activity and toxicity of MJ-III-65 (10-50 mg/kg) and NSC 314622 (5-10 mg/kg) administered i.v. push once a week for 4 weeks in nude mice bearing human head and neck xenografts of A253 and FaDu. Although these agents have limited solubility, both agents were moderately active against human A253 and FaDu tumor xenografts without significant toxicity. NSC 314622 shows about 65% tumor growth inhibition and delayed tumor growth (increased tumor doubling time). MJ-III-65 was slightly more active with similar response rate against both A253 and FaDu tumor xenografts. Defining accurately the potential therapeutic activity of these compounds is perhaps limited by the limited solubility of these compounds relative to other Top1 poisons like irinotecan.

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Discussion

Work on design and synthesis of potent Top1 inhibitors that overcome the limitations of CPT and its derivatives has been ongoing. Non-CPT Top1 inhibitors like the indolocarbazoles NB-506 and J-107088 are in clinical trials (Meng et al., 2003). More recently, indenoisoquinolines and minor groove binders (benzimidazoles) have been reported to be promising Top1 inhibitors (Cushman et al., 2000; Fox et al., 2003; Jayaraman et al., 2002; Nagarajan et al., 2003; Rangarajan et al., 2000; Strumberg et al., 1999). Preliminary screening of approximately 100 derivatives of our parent indenoisoquinoline compound NSC 314622 has identified MJ-III-65 (NSC 706744) to be a potent inhibitor of purified Top1 in biochemical assay (Antony et al., 2003; Cushman et al., 2000). This study was carried out to further characterize and determine the molecular mechanism by which MJ-III-65 exhibits its cytotoxicity in cellular systems.

Our results indicate that both the parent compound NSC 314622 and MJ-III-65 trap Top1 at similar sites with MJ-III-65 being more potent and producing Top1 cleavage complexes even at the lowest concentration of 30 nM (see Fig. 2A). MJ-III-65's potency, which increases and saturates with dose, is comparable with that of CPT though differing in the sequence/cleavage sites preferred.

We find that MJ-III-65 also inhibits Top1 in human leukemic cells as Top1-DNA complexes were detected by the ICE bioassay and by alkaline elution for DPC and SSB (Table 1 and Figs. 3 & 4). Therefore, MJ-III-65 traps Top1 both *in vitro* and in cells.

While CPT derivatives are also potent Top1 inhibitors in cells, they are limited by the rapid reversibility of the cleavage complexes upon drug removal, imposing prolonged drug treatments. On the other hand, MJ-III-65-induced Top1-cleavage complexes in cells persisted after drug removal indicating stability of the MJ-III-65-induced Top1-DNA complexes (Fig. 5). This is also supported by the *in vitro* data (Antony et al., 2003), where Top1-mediated DNA cleavage complexes trapped by MJ-III-65 are more stable (~4-fold) than those induced by CPT. Moreover, MJ-III-65 enhanced the DNA cleavage rate of Top1 (~2-fold) more than CPT. Hence, we propose that MJ-III-65 binding more tightly to the Top1-DNA cleavage complex could account for the prolonged stability of the MJ-III-65-induced DNA-Top1 complexes in living cells. Like the homocamptothecins BN80915 and BN80927 (Demarquay et al., 2004; Philippart et al., 2000), MJ-III-65 stabilizes Top1-DNA cleavage complexes to a greater extent after drug removal as compared to CPT but differs from BN80927 that can also inhibit Topoisomerase II-mediated DNA relaxation (Demarquay et al., 2004).

Another frequently encountered therapeutic limitation of CPT derivatives is drug resistance. CPT-resistant (Fig. 6B) human leukemic cells CEM/C2 with a point mutation in Top1 (Fujimori et al., 1995; Fujimori et al., 1996) were sensitive to MJ-III-65 even at 0.1 μM drug concentration (see Fig. 6A). The murine P388/CPT45 cells that do not have any detectable Top1 were also sensitive to MJ-III-65 though at higher doses (> 1 μM, Fig. 7A). This implies that MJ-III-65 has additional targets beside Top1 at high concentrations. As shown previously (Cushman et al., 2000), MJ-III-65 intercalates with DNA at high concentrations.

The ability to bind DNA could account for the additional targets that mediate MJ-III-65 cytotoxicity.

Both the parent NSC 314622 and the derivative MJ-III-65 are active against human head and neck tumor xenografts of A253 and FaDu with about 64-72% tumor growth inhibition (from Table 2 and Figs. 8 and 9) compared to untreated control in spite of their limited solubility. In xenografts bearing FaDu and A253 tumors, the response to the maximum tolerated dose of irinotecan (CPT-11, 100 mg/kg/wk x 4) is 30% and 0% complete tumor regression, respectively. In contrast, these tumors were resistant to Topotecan. MJ-III-65 is slightly more potent in terms of tumor inhibition as compared to NSC 314622, which may be, at least in part due to its higher solubility and higher dose (10 mg/kg for NSC 314622 versus 50 mg/kg for MJ-III-65). Because the toxicity (animal body weight loss) is very minimal in the highest tested dose with both agents increasing the bioavailability by improving the solubility or changing the route of administration may increase the efficacy of the drugs.

Based on the stability of MJ-III-65-induced Top1-DNA cleavage complexes and its ability to remain active even in CPT-resistant cell lines, MJ-III-65 is a promising non-CPT Top1 inhibitor whose therapeutic potential should be further explored.

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Footnote

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Legends for figures

Fig. 1. Structures of two indenoisoquinolines. NSC 314622 was discovered first. MJ-III-65 is the derivative studied in this report.

Fig. 2. Reversible trapping of Top1 by MJ-III-65.

A, The DNA corresponds to the 3'-end-labeled *Pvu*II/*Hind*III fragment of pBluescript SK(-) phagemid DNA (pSK). DNA fragments were reacted with Top1 in the absence of drug (Top1) or presence of the indicated concentrations (μM) of CTP, NSC 314622 or MJ-III-65. Reactions were at 30°C for 20 min and stopped by adding 0.5% sodium dodecyl sulfate. DNA fragments were separated in 16% denaturing polyacrylamide gels. **B**, The 3'-end-labeled pSK DNA used was the same as in Fig. 2A. Reactions were performed with Top1 in the absence of drug (Top1) or in the presence of 1 μM CPT (CPT) or 1 μM MJ-III-65 (MJ-III-65). After incubation at 30°C for 20 min, reactions were reversed by adding NaCl to a final concentration of 0.35 M for the indicated time points (min) before addition of 0.5% sodium dodecyl sulfate and proteinase K digestion. Time 0 refers to the samples taken immediately before NaCl addition. DNA fragments were then separated in a 16% denaturing polyacrylamide gel. Arrows indicate cleavage sites that are unique to CPT (◂) or unique to indenoisoquinolines (◁) or common to both (◀). Numbers to the side of the gels indicate the migration position of DNA fragments cleaved at this position in the DNA fragment analyzed.

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Fig. 3. MJ-III-65 induces Top1 cleavage complexes in CEM cells.

CEM cells were treated with 1, 10 and 100 µM MJ-III-65; 1 µM CPT; or 100 µM VP-16 for 6 hr at 37^{o}C. Equal number of cells were lysed in 1% sarkosyl and submitted to the ICE assay (see "Materials and Methods"). DNA-containing fractions were collected from the bottom of the gradients. Fractions (7-10) were blotted and Top1-DNA and Top2-DNA covalent complexes were detected using Top1 C21 monoclonal and Top2 antibody. Treatment with 1 µM CPT and 100 µM VP-16 was used as positive control for Top1 and Top2 cleavage complexes respectively.

Fig. 4. DNA-protein crosslinks induced by MJ-III-65 in human leukemic CEM cells.

Cells were prelabeled with [^{3}H]-thymidine and were treated with 0.03, 0.1, 0.3, 1, 10 or 100 µM MJ-III-65 for 1 hr at 37^{o}C. DPC were assayed by alkaline elution. Results from three different experiments (■ , ○, △) are plotted.

Fig. 5. Persistence of DNA-Protein crosslinks (DPC) induced by MJ-III-65 in human leukemic CEM cells after drug removal.

Cells were prelabeled with [^{3}H]-thymidine and were treated with either 1 µM MJ-III-65 or 1 µM CPT for 1 hr at 37^{o}C. DPC were assayed immediately before drug removal (T_0) and after drug treatment followed by culturing in drug free medium for 30 min (T_{30}) or 60 min (T_{60}) respectively. DPC induced in MJ-III-65 and CPT treated cells (●) are plotted as an average of two independent experiments ± SD.

Elution of untreated cells (○) is also shown.

Fig. 6. Sensitivity of CPT-resistant Top1-mutant CEM/C2 cells to MJ-III-65. Growth inhibition in CEM (●) and CEM/C2 (○) cells was measured by MTT assay after treatment with MJ-III-65 (A) or CPT (B) for 1 hr. MJ-III-65 and CPT concentrations used were as follows: 0.01, 0.1, 1 and 10 μM. Percent growth of two independent experiments is represented as the mean ± SD.

Fig. 7. Resistance of Top1-deficient P388/CPT45 cells to MJ-III-65. Growth inhibition in P388 (●) and P388/CPT45 (○) cells were measured by MTT assay after treatment with 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 μM of MJ-III-65 (A) or CPT (B) for 3 days. Percent growth of two independent experiments is represented as the mean ± SD.

Fig. 8. Antitumor activity (A) and toxicity (B) of NSC 314622 in nude mice bearing human A253 and FaDu head and neck tumor xenografts. Control (untreated) (●); NSC 314622 5 mg/kg (▼); NSC 314622 10 mg/kg (■)

Fig. 9. Antitumor activity (A) and toxicity (B) of MJ-III-65 in nude mice bearing human A253 and FaDu head and neck tumor xenografts. Control (untreated) (●); MJ-III-65 10 mg/kg (▼); NSC MJ-III-65 25 mg/kg (▲); NSC 314622 50 mg/kg (■)

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Table 1. Estimated DNA-protein crosslinks (DPC) and single strand breaks (SSB) in MJ-III-65 treated CEM cells. For comparison, DPC in CPT-treated cells was determined. Values obtained from independent experiments are listed. NT: not tested.

Drug	Concentration (μM)	DPC (Rad-Eq.)	SSB (Rad-Eq.)
Untreated Control	0	0	0
MJ-III-65	0.03	403, 433	378, 579
MJ-III-65	0.1	845, 913	527, 894
MJ-III-65	0.3	1274, 1288, 1410	531, 834
CPT	0.3	1720	NT

DNA lesion frequencies are expressed in rad-equivalents (Kohn, 1991; Ross et al., 1979). In case of SSB, one rad-equivalent corresponds to approximately 1 SSB/10^9 nucleotides.

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Table 2. Antitumor activity and toxicity of NSC 314622 and MJ-III-65 in nude mice bearing human A253 and FaDu head and neck xenografts.

Treatment (mg/kg/wk)	**MWL** (%)	**MTGI** (%)	**TDT** (Day)
A253			
Control	0	--	3.4 ± 0.4
NSC 314622 **(5)**	3.6 ± 2.2	64.2 ± 3.2	7.8 ± 0.7
NSC 314622 **(10)**	3.4 ± 1.1	65.6 ± 4.2	8.0 ± 0.9
MJ-III-65 **(10)**	2.8 ± 2.0	56.1 ± 11.4	8.2 ± 1.2
MJ-III-65 **(25)**	4.9 ± 1.9	66.8 ± 7.9	8.8 ± 1.0
MJ-III-65 **(50)**	5.2 ± 1.6	71.8 ± 4.2	9.2 ± 1.2
FaDu			
Control	0	--	3.2 ± 0.5
NSC 314622 **(5)**	3.6 ± 2.2	63.5 ± 4.4	7.9 ± 1.0
NSC 314622 **(10)**	3.4 ± 1.1	66.4 ± 5.2	8.2 ± 1.5
MJ-III-65 **(10)**	2.8 ± 2.0	56.6 ± 10.7	8.1 ± 1.0
MJ-III-65 **(25)**	4.9 ± 1.9	66.2 ± 5.2	8.6 ± 0.8
MJ-III-65 **(50)**	5.2 ± 1.6	69.0 ± 3.5	9.0 ± 1.0

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MWL: maximum weight loss of pretreatment body weight; MTGI: maximum tumor growth inhibition; TDT: tumor doubling time.

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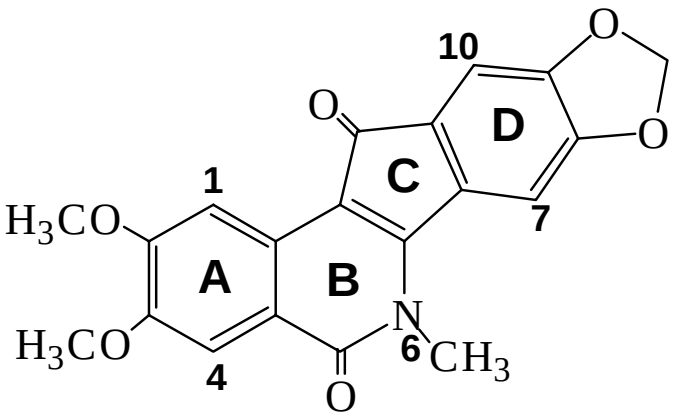
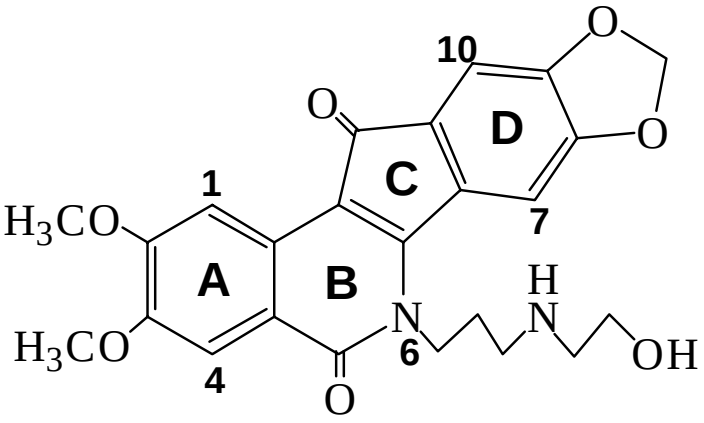
Fig 1

NSC 314622



MJ-III-65



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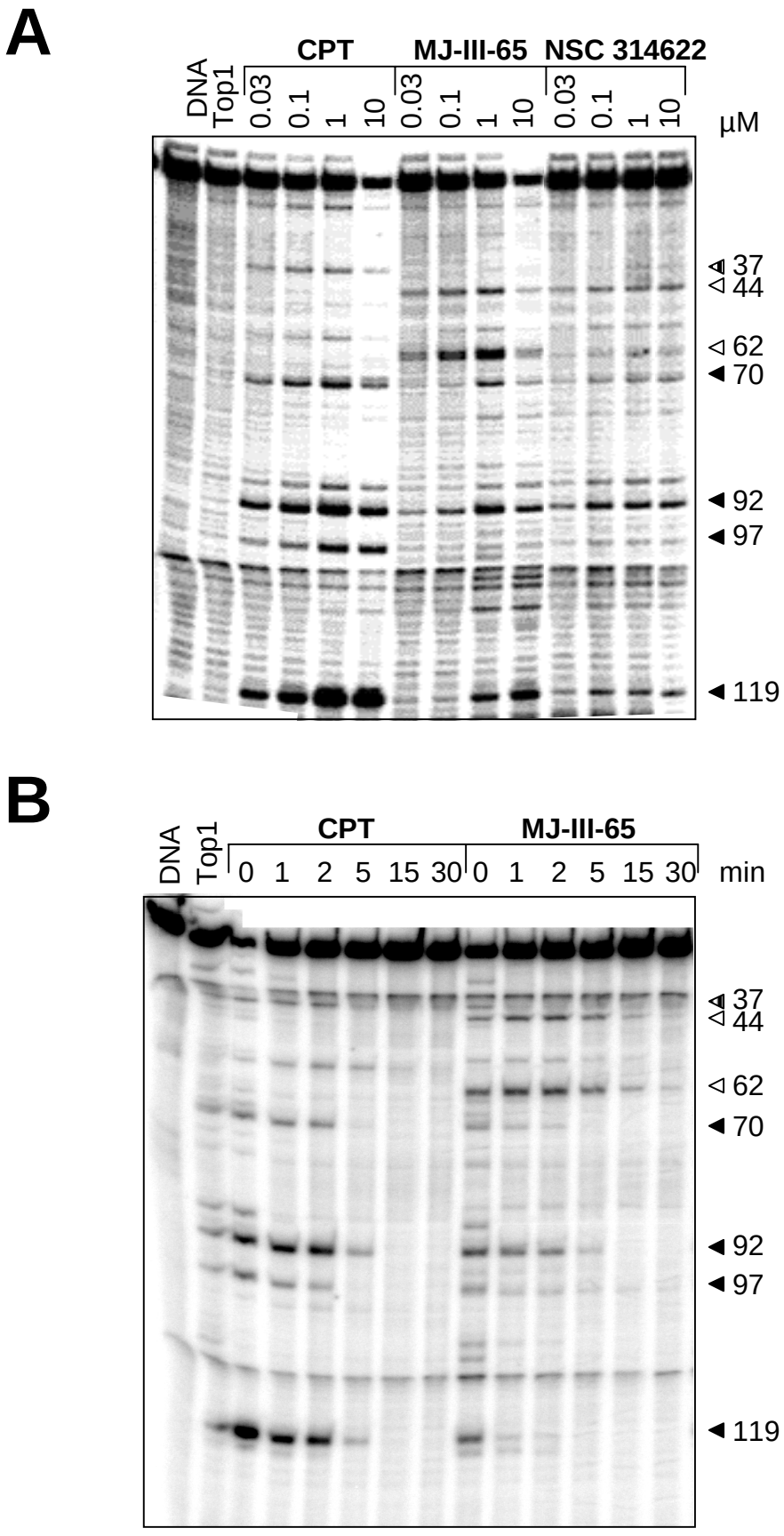

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Fig 3

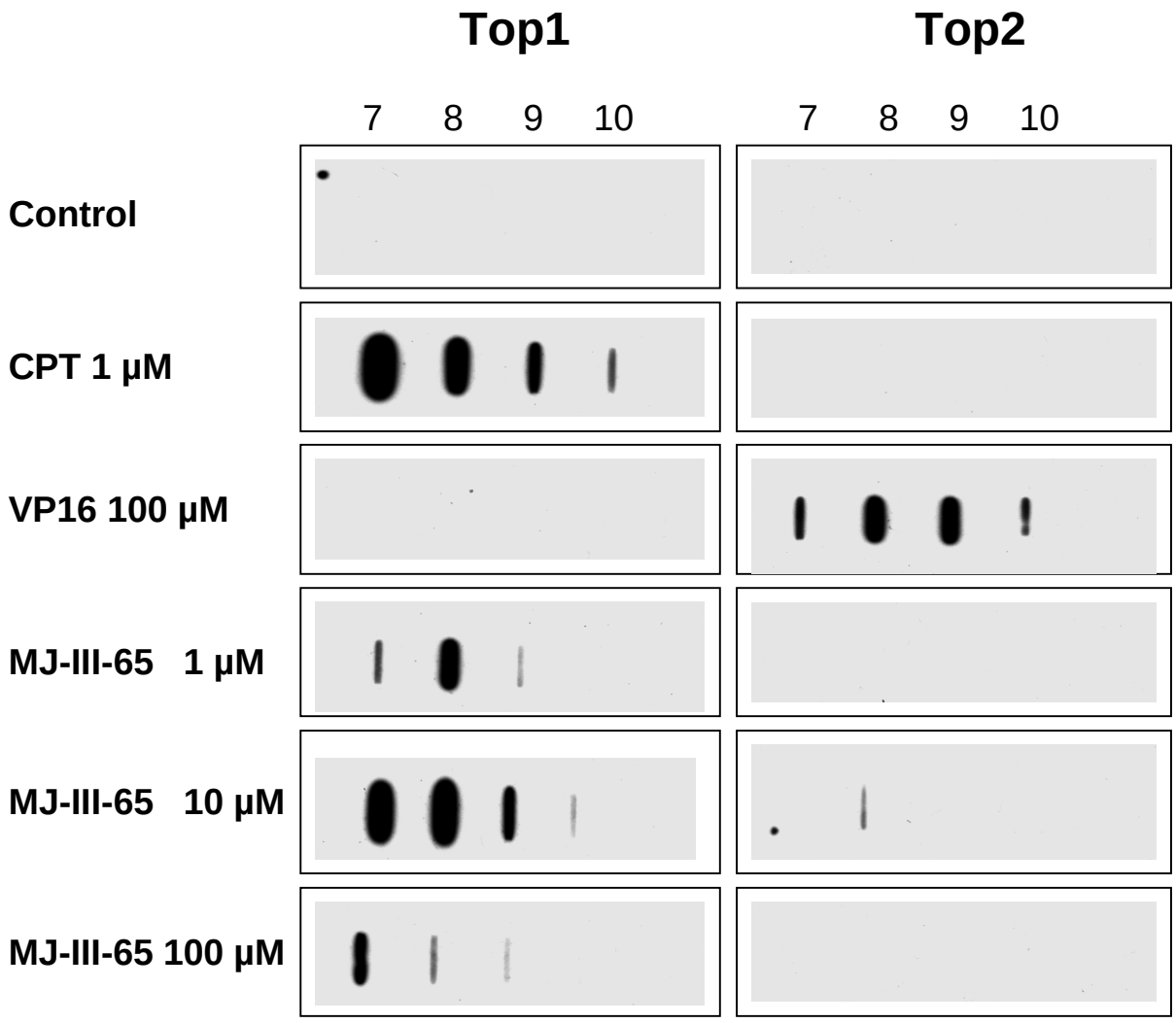

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Fig 4

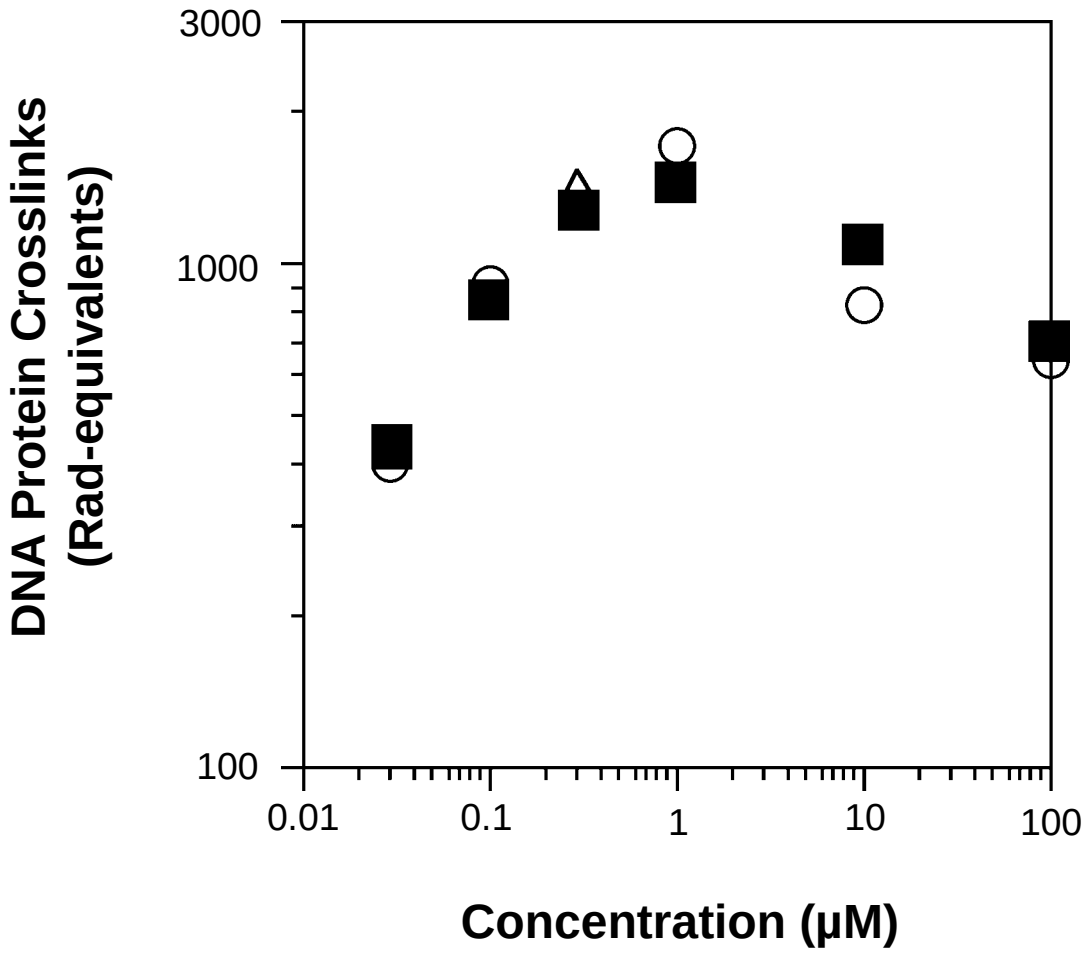

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Fig 5

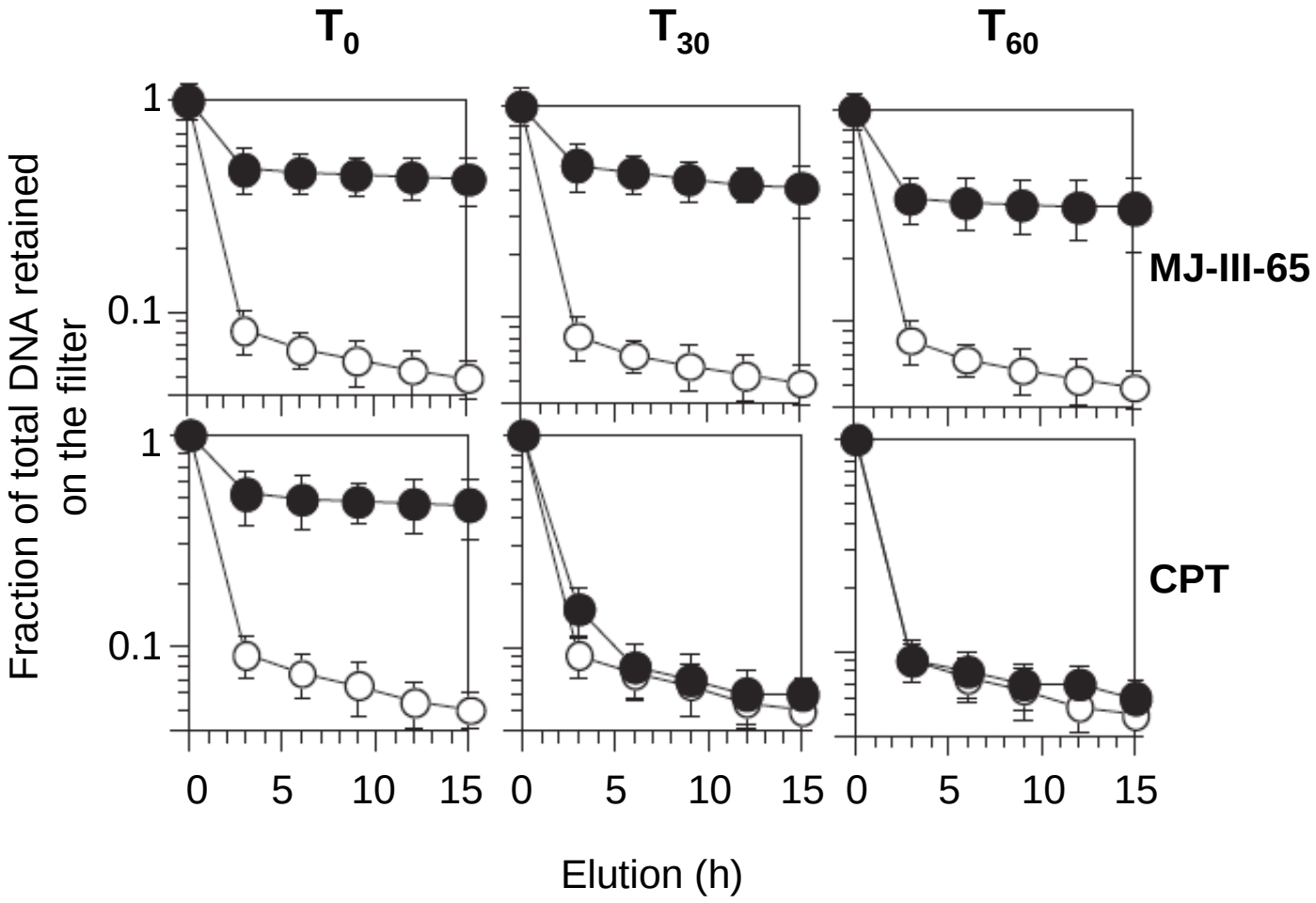

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Fig 6

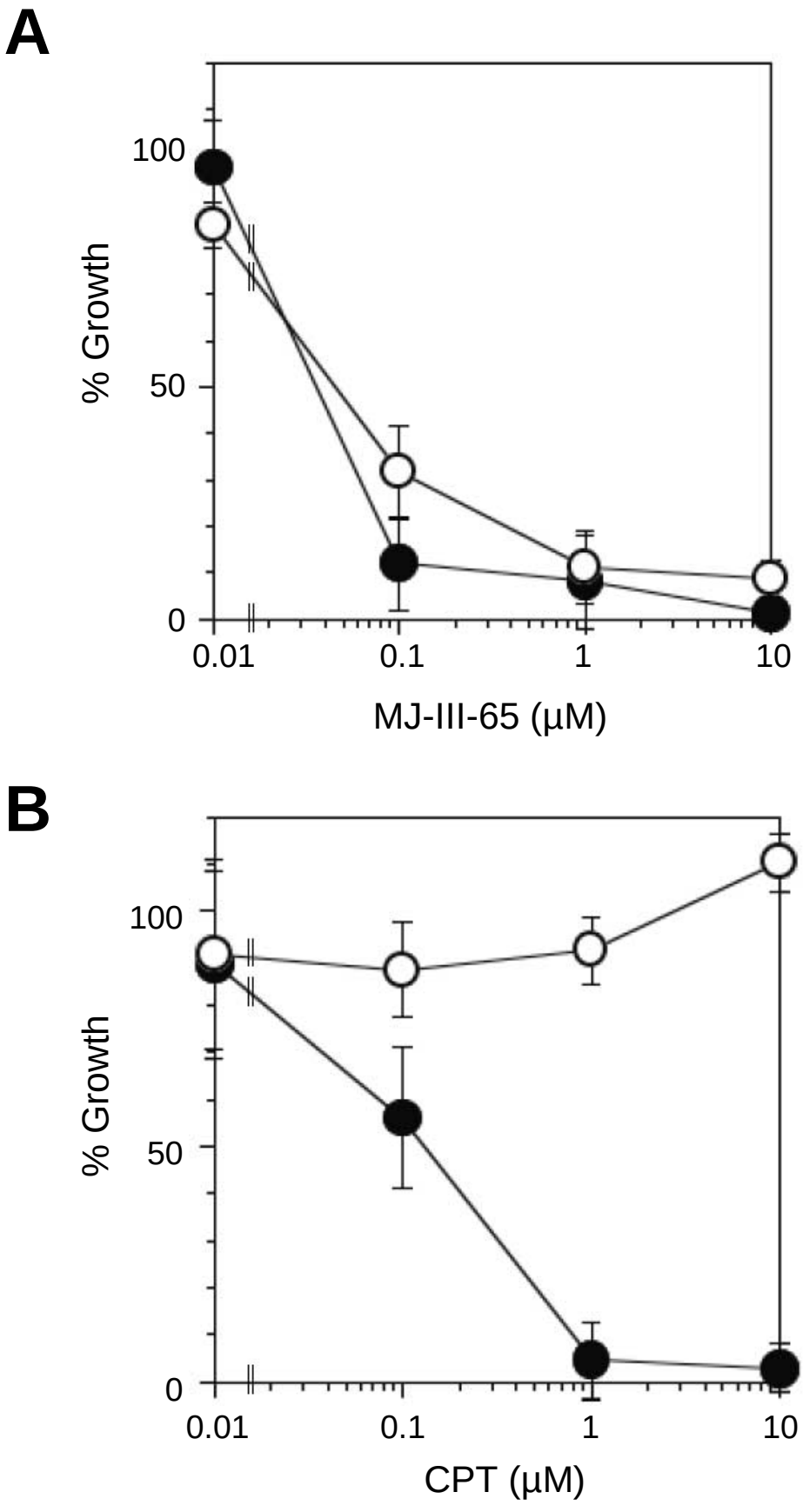

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Fig 7

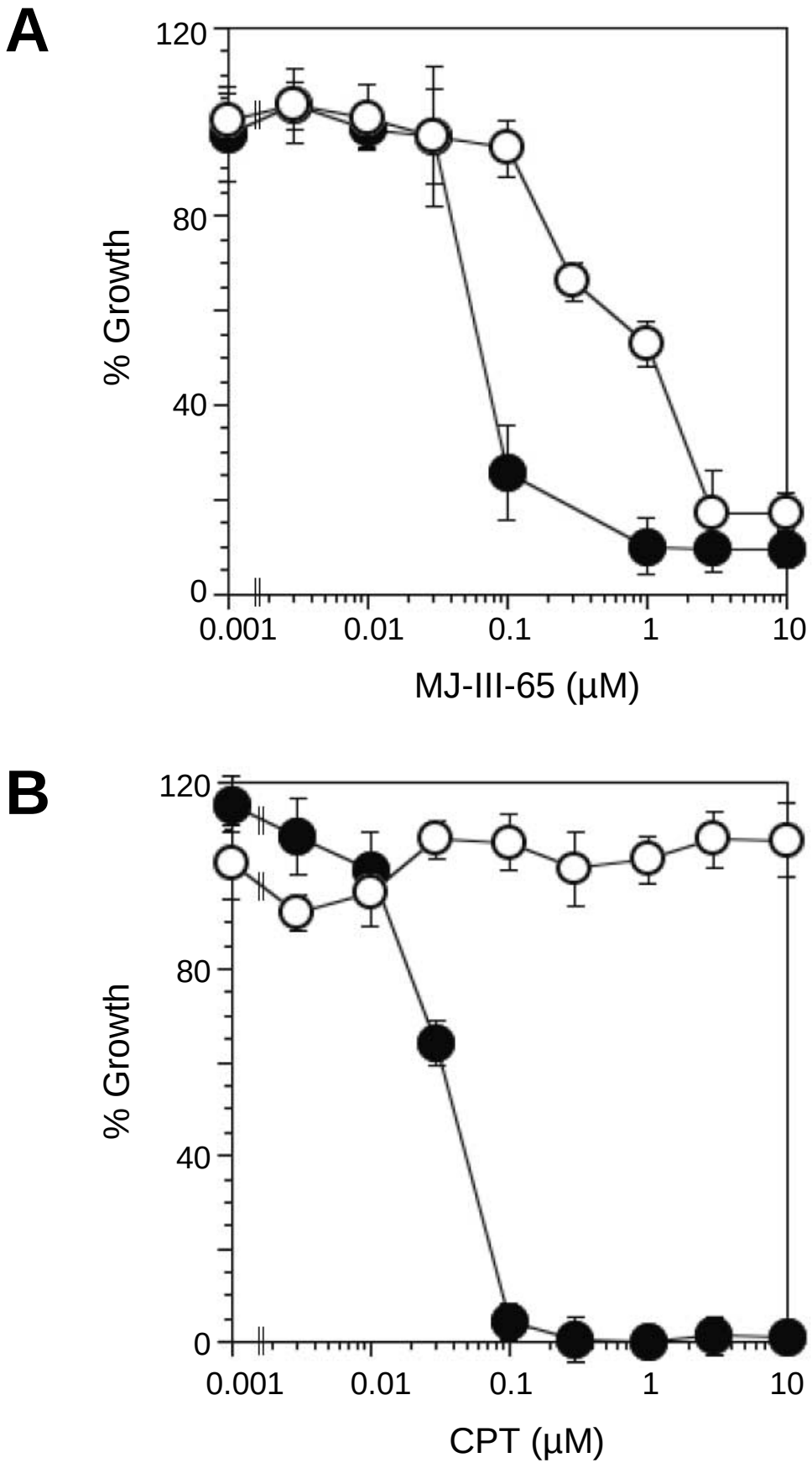

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Fig 8

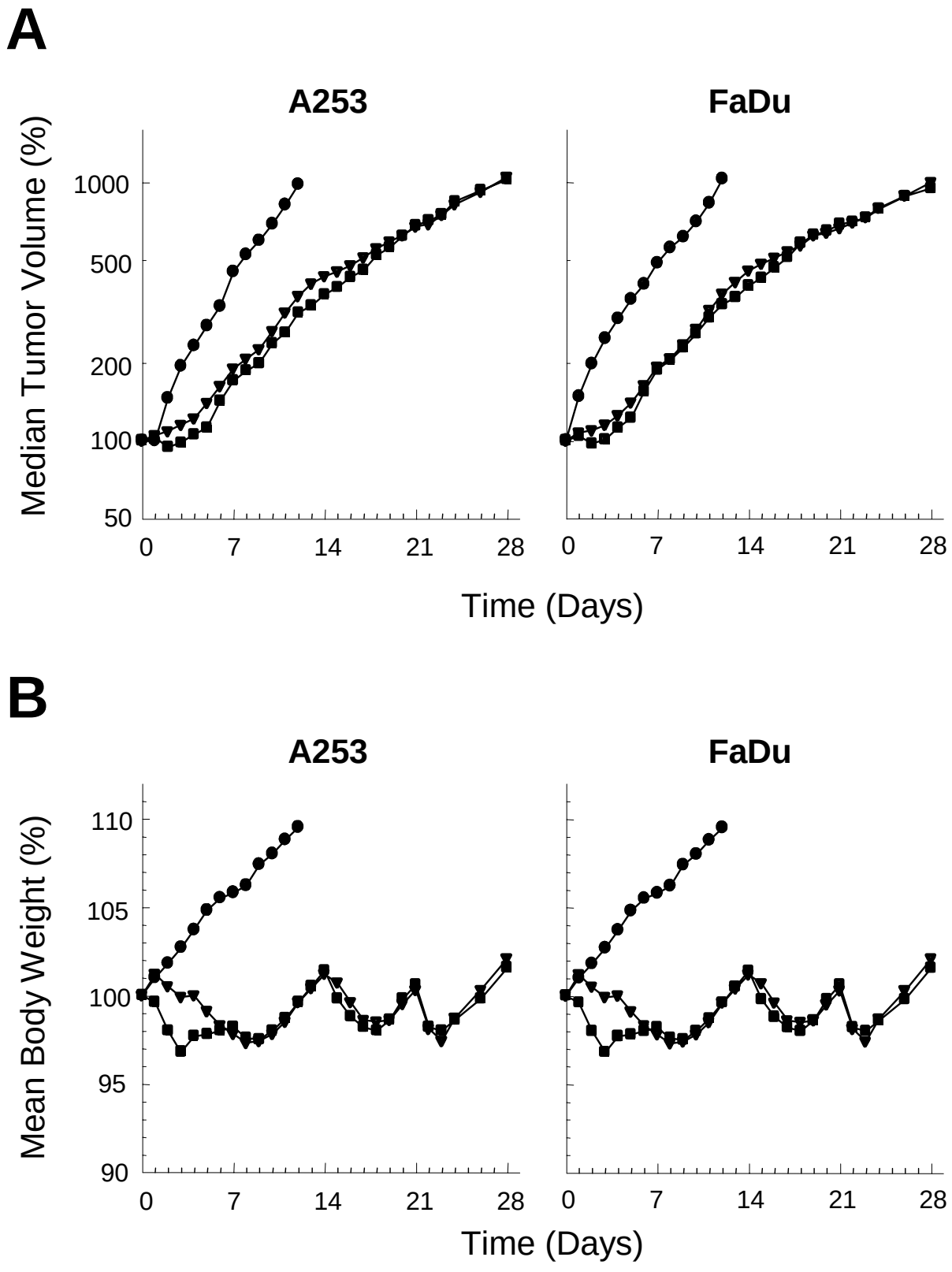

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Fig 9