JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053

XRCC3 induces cisplatin resistance by stimulation of Rad51-related recombinational repair, S-phase check point activation, and reduced apoptosis.

Zhi-Yuan Xu, Martin Loignon, Fei-Yu Han, Lawrence Panasci and Raquel Aloyz

Lady Davis Institute for Medical Research, Sir Mortimer B. Davis - Jewish General Hospital, Montréal, Quebec, Canada, ZYX, ML, LP and RA. Pathology and Human Genetics, McGill University and Cytogenetics, McGill University Hospital Center, Montreal Children's Hospital, FYH

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

a) Running title: Xrcc3 mediates cisplatin resistance

b) Corresponding author: Dr. Lawrence Panasci. Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, 3755 Cote Ste Catherine Road, Montreal, Quebec, Canada H3T 1E2. Phone: (514) 340-8222 ext 5281; fax: (514) 340-8302; e-mail: lpansasci@hotmail.com

c) Number of text pages: 24

Number of tables: 0

Number of figures: 9

Number of references: 30

Number of words in the

Abstract: 203

Introduction: 623

Discussion: 778

d) List of non standard abbreviations used

HRR, homologous recombinational repair

MLN, melphalan

CDDP, cisplatin

ICLs, interstrand crosslinks

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

NER, nucleotide excision repair

NHEJ, non-homologous end-joining

DSBs, double strand breaks

e) Recommended section assignment

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Eukaryotic cells respond to DNA damage by activation of DNA repair, cell-cycle arrest and apoptosis. Several reports suggest that such responses may be coordinated by communication between damage repair proteins and proteins signalling other cellular responses. The Rad51-guided homologous recombination repair system plays an important role in recognition and repair of DNA interstrand crosslinks (ICLs) and cells deficient in this repair pathway become hypersensitive to ICL inducing-agents such as cisplatin and melphalan. We investigated the possible role of the Rad51-paralog protein, Xrcc3, in drug resistance. Xrcc3 overexpression in MCF-7 cell resulted in: (a) a 2-6 fold resistance to cisplatin/melphalan, (b) a 2 fold increase in drug-induced Rad51 foci, (c) an increased cisplatin-induced S-phase arrest (d) decreased cisplatin-induced apoptosis and (e) increased cisplatin-induced DNA synthesis arrest. Interestingly, Xrcc3 overexpression did not alter the doubling time or cell cycle progression in the absence of DNA damage. Furthermore, Xrcc3 overexpression is associated with increased Rad51C protein levels consistent with the known interaction of these two proteins. Our results demonstrate that Xrcc3 is an important factor in DNA cross-linking drug resistance in human tumor cells and suggest that the response of the homologous recombinational repair machinery and cell cycle checkpoints to DNA crosslinking agents is intertwined.

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Cisplatin is one of the most potent antitumor agents known displaying clinical activity against a wide variety of tumors. Its cytotoxicity is mediated by the induction of DNA intrastrand adducts and DNA interstrand cross-links (ICLs). ICLs are particularly deadly because a single unrepaired ICL impairs DNA replication and translation. The repair of ICLs is through to involve the combined action of nucleotide excision (NER) repair components, which unhooks the ICL; either initially or after strand invasion, along with the action of the homologous recombinational repair (HRR) machinery. In cycling mammalian cells, HRR is a major pathway involved in the repair of double strand breaks (DSBs), which are probably produced during the ICL repair process, while non-homologous end-joining (NHEJ) is favoured when recombinational substrates are not available (G1 phase of the cell cycle) (De Silva et al., 2000; McHugh et al., 2001; West, 2003). The HRR process requires the assembly of multienzymatic complexes visualized immunocytochemically as Rad51 nuclear foci. These complexes include the Rad51 paralog family members such as Rad51, Rad54, Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3 (reviewed in West, 2003). Rad51 paralog defective cell lines (Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3) present similar phenotypes: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents and attenuated Rad51 focus formation after exposure to ionizing radiation (IR) (Takata M et al, 2001; Liu et al, 1998). Recently similar results has been obtained in a human colon cancer cell line in which Xrcc3 was inactivated by gene targeting (Yoshihara, 2004).However, in these cells, Xrcc3 deficiency results in milder sensitivity to DNA cross-linking agents (two fold) when compared with previous results in hamster deficient cells. Moreover Xrcc3 deficiency resulted in increased endoreduplication.

Rad51C is involved in at least two complexes; one containing Rad51B-Rad51C-Rad51D-Xrcc2 and another containing Rad51C-Xrcc3 (Masson, 2001). Rad51C and Xrcc3 may be involved in Holliday

junction resolution while other Rad51 paralog members may be involved in branch migration processes (Liu, 2004).

One of the biological responses to DNA damage is to slow progression through S-phase as a consequence of activating a checkpoint. For example, following exposure to ionizing radiation (IR), cells activate the ATM kinase, which initiates a generalized response that includes the S-phase checkpoint pathway to delay DNA replication and allows repair of DNA. In response to DSB induction, ATM triggers two parallel cascades that cooperate to inhibit DNA replication, resulting in the S-phase checkpoint. The two parallel cascades involve ATM-dependent phosphorylation of Chk2 and Nbs1, respectively. The DNA damage-activated kinases, Chk1 and Chk2, are also implicated in regulation of G_2 checkpoints (Falck et al, 2002). p53 has been shown as well to be an important component of G_1 and G_2 arrest in response to DNA damage. The p53-responsive p21 protein inhibits cyclin-dependent kinases (CDKs) that drive cell cycle progression. p53-dependent p21 induction is thought to mediate G_1 and G_2 arrest in response to DNA. Even though the IR-induced-S-phase checkpoint summarized above has been extensively described, little is known about the molecular mechanisms involved in the S-phase checkpoint elicited in mammalian cells by DNA crosslinking agents. Enhanced DNA repair of ICLs produced by DNA crosslinking agents has been associated with resistance to these agents (Torres-Garcia et al, 1989; Batist et al, 1989; Spanswick et al, 2002). Moreover, increased Xrcc3 protein levels in cell lines and clinical samples correlated with DNA crosslinking agent resistance (Wang et al, 2001; Bello et al, 2002).

In the present study we investigate the consequences of the Rad51 related paralog, Xrcc3 overexpression in terms of cell cycle progression, Rad51-related homologous recombinational repair and cell survival after cisplatin treatment in the breast cancer cell line, MCF-7. Our results demonstrate that Xrcc3 mediates cisplatin resistance by a Rad51-dependent mechanism and suggest that cross-talk between HRR and cell cycle check points may exist.

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

METHODS

Cell Culture and Stable Transfection: MCF-7 cells were maintained as described (Batist et al. 1989). The Xrcc3 open reading frame sequence was sub-cloned into the pcDNA3.0 expression vector (Invitrogen), amplified, and stably transfected into the human breast cancer cell line, MCF-7, using the Effectine reagent (Qiagen) following the manufacturer's instructions. The transfected cells were maintained in medium for 36 h, trypsinized and serially diluted. Single clones were amplified for three weeks in medium containing 600 μg/ml of G418. Mock-transfected MCF-7 cells were obtained by transfection of the empty pcDNA3.0 expression vector.

Cell Survival Assay: The cells were seeded in 96 well-plates until 50% confluent, and then treated with cisplatin (CDDP, 0-100μM) (Mayne, Montreal, Quebec, Canada) or melphalan (MLN, 0-100μM) (Sigma-Aldrich). Survival was assessed 7 days after treatment using the SRB colorimetric assay described previously (Batist et al, 1989; Bello, et al, 2002; Aloyz et al, 2002). The IC_{50} (concentration of drug) that results in 50% of control was calculated as previously described (Batist et al, 1989; Bello, et al, 2002; Aloyz et al, 2002). The IC_{50} values represent the mean and the 95% confidence intervals (CIs) of three independent experiments.

Annexin V Assay: Subconfluent cultures were treated with CDDP (0 or 20μM) and the induction of apoptosis was determined as described before (Aloyz et al 2004) using the Annexin V-EGF (Clonotech). Briefly, 0-36 hours after treatment, floating and adherent cells were harvested, washed with PBS, fixed and stained following the manufacturer instructions. 7-AAD (BD Pharmingen) was utilized to discriminate apoptosis from necrosis. The cells were immediately subjected to bivariate analysis using a FACSCalibur flow cytometer (Becton Dickinson). The percentage of annexin V cells represents the mean value and the 95% intervals (CIs) of two independent experiments.

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

FACS Analysis: Subconfluent cultures were treated with CDDP (0 or 20μM) for 1 hour and the DNA content was determined by FACS analysis using propidium iodide as described before (Aloyz et al, 2002). Cell cycle analysis was performed using a fluorescent-activated cell sorter (EPICS XL-MCL, Beckman/Coulter). The percentages represent the mean value and the 95% intervals (CIs) of two independent experiments.

Rad51 Foci Density Determination: Rad51 foci density was determined as described previously with minor modifications (Wang et al, 2001; Aloyz et al, 2002). At various time points after 1 hour exposure to 20 μM CDDP- treatment, the cells were washed with PBS fixed and stained with a specific Rad51 rabbit antibody (H-92 Santa Cruz Biotechnology). A fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (Santa Cruz Biotechnology) was used as secondary antibody and the nuclei were counterstained with propidium iodide (PI, Sigma Chemical Co., St. Louis, MO). Changes in Rad51 nuclear density were determined by confocal microscopy as described before. The merged images with yellow staining represent the Rad51nuclear localization. The total Rad51 foci density was determined as the ratio of the average yellow intensity (fluorescent total intensity, FTI) relative to the total yellow (fluorescent total area, FTOA)-stained area in five randomly selected fields or nuclei for each treatment.

Sister Chromatid Exchanges (SCEs): Sister chromatid exchanges/cell were determined after treatment with cisplatin (0μM, 0.5μM or 2.5μM) as described before (Aloyz et al, 2002). Twenty-four hrs after seeding, OVER and MOCK cells were treated with cisplatin. One hour after cisplatin treatment, fresh medium containing 0.04 ug BrdUrd/ml (Boehringer Mannheim) was added to the cultures for 44 hrs (two doubling times). During the final 5h of culture, mitotic cells were arrested in metaphase with 0.01 ug /ml Colcemid (Life Technologies, Inc.). Metaphase preparation was done by

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

standard cytogenetic procedures. Differential sister chromatid staining was achieved by the fluorescence-plus-Giemsa method (Aloyz et al, 2002). Enumeration of SCEs was done without knowledge of treatment in 10 well-spread second-division metaphases for each culture.

Western Blot Analysis: Western blot analysis was performed using specific antibodies to α-Xrcc3 (1/1000 dilution; a kind gift of Dr. P. Sung, Howard Hughes Medical Institute, Department of Molecular Biology, USA), p53, p21 (1/2000 dilution, neomarkers USA), phosphorylated Chk2 (Cell Signaling), ERK2 (Santa Cruz), α-tubulin (1/10,000 dilution; Medicorp), Rad51 C (1/1000 dilution; abcam 7898) and Rad51B (1/500 dilution; a kind gift of Dr Jean-Ives Masson, Laval University, Laval, Quebec, Canada) as described (Wang et al, 2001; Aloyz et al, 2002).

Cisplatin Resistant DNA Synthesis: The cells were plated in 6 well dishes and cultured for 2 days, until 70% confluence, in the presence of 15 nCi/ml [^{14}C] thymidine to label total cellular DNA. Fresh medium with [^{14}C]thymidine was utilized 24 hours after initial plating. Following 2 days, the cells were treated with 10μM CDDP or vehicle. One hour after treatment the medium with cisplatin was removed and drug-free media was added. At different times after cisplatin removal (2-36h), fresh medium with 6 μCi/ml of [^{3}H]methylthymidine was added for 1 hour. Following aspiration of the medium and two washes with ice-cold 5% TCA, the cells were solubilized in 0.3 N NaOH. An aliquot of neutralized (with glacial acetic acid) NaOH solubilized material was added to a scintillation vial and the ^{3}H and ^{14}C radioactivity determined by dual channel liquid scintillation counting. The relative rates of DNA synthesis were determined by calculating the ratio of ^{3}H dpm:^{14}C dpm. The rates of DNA synthesis were expressed as a percentage of control, i.e.; the ^{3}H:^{14}C ratio in cells receiving vehicle treatment (Guo et al, 2002).

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

Statistical Analysis

The results are expressed as the mean values ± 95% confidence intervals. Differences between mean values were assessed using the two tailed, paired t-test for means.

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS:

XRCC3 MEDIATES CDDP AND MLN RESISTANCE

Two Xrcc3 transfected MCF7 clones, **OVER1** and **OVER 2,** overexpress Xrcc3 by 2 and 4 fold respectively when compared to mock transfected cells (**MOCK** thereafter) [Figure 1A]. The resistance of CDDP and MLN in these cells correlates with Xrcc3 protein levels (r = 0.95) [Figure 1B]. The Xrcc3 overexpressing clones displayed resistance to CDDP (**OVER1** 3.4 fold and **OVER2** 4.4 fold) and MLN (**OVER1** 2.5 fold and **OVER 2** 4 fold) when compared to the **MOCK** cells [Figure 2 AB]. The results suggests that Xrcc3 is an important factor in DNA cross-linking agent cytotoxicity since Xrcc3 protein expression correlates with increasing CDDP/MLN resistance. We wanted to determine next if the differences in drug resistance between **MOCK** and **OVER2** cells (**OVER** thereafter) is due to a difference in CDDP-induced programmed cell death. We assessed differences in Annexin V expression between **MOCK** and **OVER** cells after CDDP treatment. We utilize a 1 hour 20 μM CDDP exposure and we assessed differences in CDDP-induced apoptosis in a two doubling time period (48 hours). In this period of time the IC_{50} determined by SRB (7 days after treatment) didn't induces detectable apoptosis. We stained **MOCK** and **OVER** cells for Annexin V 0-36 hour after CDDP treatment (Figure 3). Our results shown that CDDP induces some apoptosis in **MOCK** and **OVER** cells at ≥ 18 hours after treatment. Significantly increased CDDP-induced apoptosis was observed in **MOCK** cells when compared to **OVER** cells 18, 24 and 36 hours after CDDP. The majority of **OVER** cells were not apoptotic at 36 hours.

XRCC3 OVEREXPRESSION AFFECTS THE HOMOLOGOUS RECOMBINATIONAL REPAIR (HRR) PROCESS.

HRR occurs as a consequence of induced or spontaneous DNA damage and can be visualized by the appearance of Rad51 nuclear foci and sister chromatid exchanges (SCEs) (reviewed in Sonoda et al, 2001). Nevertheless, while Rad51 foci occur during S-phase of the cell cycle, the SCEs are the

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

reflection of postreplicational repair associated with crossing-over between sisters duplexes (Sonoda et al, 1999). Because Xrcc3 null cells are hypersensitive to cisplatin and display deficient spontaneous or induced Rad51 foci and SCEs, we investigated the effect of Xrcc3 expression levels on Rad51 foci formation and SCEs before and after 10 μM CDDP treatment (West 2003; Bishop et al, 1998). Rad51 foci nuclear density was similar in **OVER** and **MOCK** cells in basal conditions (data not shown). However, 12 hours after treatment when significant CDDP-induced apoptosis is not observed, drug-induced Rad51 foci density [Figure 4], expressed either as density by field [Figure 4 B] or by nucleus [Figure 4 C], was increased by 2 fold in **OVER** cells with respect to **MOCK** cells. In contrast, there was no real difference in the percentage increase of SCEs/cell induced by CDDP between **OVER** and **MOCK** cells [Figure B]. However, surprisingly, Xrcc3 overexpression resulted in a 2 fold decrease in SCEs in the absence of induced DNA-damage [Figure C].

XRCC3 OVEREXPRESSION ALTERS THE CDDP-INDUCED S-PHASE CHECKPOINT

Xrcc3 overexpression did not affect the doubling time of MCF-7 cells (22h), suggesting that even though Xrcc3 expression is required for normal cell division, Xrcc3 overexpression does not alter this process in a Rad51 proficient background (Tebbs et al, 1995). Similarly, there were no significant differences between **OVER** and **MOCK** cells in the FACS profile in basal conditions [Figure 6 left panel] suggesting that cell cycle progression is not affected by Xrcc3 overexpression in the absence of induced DNA damage. Early after CDDP treatment, there were not significant differences in cell cycle progression between **MOCK** and **OVER** cells. Six hours after CDDP treatment, **MOCK** and **OVER** cells undergo S-phase arrest [Figure 6 central panel]. However, 12 hour after treatment, a significantly higher percentage of **OVER** cells were arrested in S-phase when compared with **MOCK** cells [Figure 6 right panel]. Since S-phase checkpoint induction involves DNA synthesis arrest to allow DNA repair before the cells undergo mitosis (Cliby et al, 1998), CDDP-induced DNA synthesis arrest in **OVER** and **MOCK** cells after CDDP treatment was examined. There was no significant difference

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

in DNA synthesis rate in the absence of CDDP treatment between the cell lines (data not shown). CDDP induced a transient DNA synthesis arrest (2-40 hours) in both cell lines. However, the DNA synthesis arrest induced by CDDP was more pronounced in **OVER** than in **MOCK** cells (1.3 to 1.5 fold) [Figure 7]. Moreover, in keeping with the role of Chk2 in S-phase arrest, CDDP-induced Chk2 phosphorylation was more sustained in **OVER** cells (6-24hour) than in **MOCK** cells (6-12 hour) [Figure 8 AB]. At a later time point (36 hours), when Chk2 phosphorylation was decreased in both cell lines to basal levels, the percentage of **OVER** cells in S-phase was higher than in **MOCK** cells [Figure 8 C].

p53 PROTEIN LEVELS CORRELATES WITH INCREASED CDDP-INDUCED APOPTOSIS IN MOCK CELLS.

We did not find differences in the inductions of p53 and p21 between **MOCK** and **OVER** in p53 protein levels 6 to 24 hours after CDDP treatment [Figure 8 AB]. However, 48 hours after treatment p53 levels were close to basal values in **OVER** cells while in **MOCK** cells p53 levels remained elevated [Figure 8 AB].

XRCC3 PROTEIN LEVELS CORRELATE WITH RAD51C PROTEIN LEVELS.

Overexpression of Xrcc3 alters Rad51C protein levels. Increasing Xrcc3 protein levels correlate with Rad51C protein levels in the **MOCK** and **OVER** cell lines [Figure 9] while Rad51B protein levels are not affected. This is consistent with the known protein-protein interaction of Xrcc3 and Rad51C (Masson et al, 2001). The increased Rad51C protein levels associated with overexpression of Xrcc3 are possibly secondary to altered protein stability.

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Different mechanisms are known to be involved in cisplatin resistance, including altered apoptosis by loss of p53 function, overexpression of bcl_2, cytoplasmic inactivation, altered transport or increased DNA repair (Siddik 2002). Our results demonstrate that Xrcc3 overexpression in MCF-7 cells results in increased survival after cisplatin treatment associated with enhanced homologous recombinational DNA repair and consequently decreased cisplatin-induced apoptosis. These results are consistent with previous reports suggesting that in primary human tumor cells and human tumor epithelial cell lines increased HRR is associated with interstrand cross-linking agent drug resistance (Torres-Garcia et al, 1989; Batist et al, 1989; Spanswick et al, 2002; Wang et al, 2001; Bello et al, 2002; Christodoulopoulos et al, 1999; Slupianek et al, 2001). However, XRCC3 overexpression in a lymphoblastoid cell line did not increase mitomycin C resistance in preliminary results (Wiese et al. 2002). The divergent results may be due to a difference in the origin of the cell lines.

The increased percentage of XRCC3 overexpressing cells arrested in S-phase 12 hours after cisplatin treatment may be the reflection of increased HRR. Alternatively and/or additionally, it is also possible that the Xrcc3-mediated S-phase arrest after cisplatin treatment reflects the activation of a checkpoint response which alters Rad51 related HRR. The observation that CDDP- induced DNA synthesis arrest and Chk2 phosphorylation is more sustained in Xrcc3 overexpressing cells than in mock transfected cells suggests that Rad51 HRR and S-phase checkpoint activation are intertwined. Our biochemical results are consistent with a scenario in which cisplatin-induced S-phase arrest in MCF-7 cells would be mediated by Chk2 phosphorylation. The sustained Chk2 phosphorylation observed in Xrcc3 overexpressing cells suggests that Xrcc3 is affecting the S-phase checkpoint pathway activated by ATM/ATR signaling pathways. Moreover, it has been reported that XRCC3 may be involved in cell cycle progression since Xrcc3 modulates replication fork progression on cisplatin damaged chromosomes via its role in homologous recombination (Henry-Mowatt et al, 2003).

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

In MCF-7 cells, cisplatin-induced apoptosis is mediated by the induction of the tumor suppressor gene p53 (Lee et al, 1999). It has been shown that in breast cancer cell lines, cisplatin-induces S-phase arrest is independent of p53 status. Accordingly, Xrcc3 overexpression did not alter p53 or p21 protein levels up to 24 hours after CDDP treatment. The late differences in p53 protein levels, 48 hours following CDDP treatment, when the surviving cells are recovering from DNA synthesis arrest, may be secondary to a higher number of Xrcc3 overexpressing cells entering mitosis after a successful DNA repair, while mock transfected cells undergo apoptosis. This hypothesis is sustained by the DNA-content profiles and the percentage of apoptotic cells observed 36 hour after treatment.

Since HHR takes place during S/G2M phases of the cell cycle, it is possible that the prolonged/increased S-phase checkpoint observed in Xrcc3 overexpressing cells is the reflection of increased cisplatin-induced DNA repair. This hypothesis is sustained by the fact that Xrcc3 overexpressing cells displayed increased Rad51 foci formation and survival after cisplatin treatment when compared to mock transfected cells. Furthermore, we have not demonstrated that Xrcc3 is necessary for activation of the S-phase check point in response to cisplatin. Also, it is possible that the effect on S-phase check point after cisplatin treatment may be due in part to differences in the stress response to DNA damage in Xrcc3 overexpressing cells. Whether or not Xrcc3 is required for the S-phase checkpoint is not known. This question could be further assessed using specific Xrcc3-siRNA inhibitors.

Increased Xrcc3 protein levels correlate specifically with increase Rad51C protein level, suggesting that that Xrcc3 overexpression stabilizes Rad51C, presumably due to increased formation of the stabilizing Rad51C-Xrcc3 heterodimer (Masson et al, 2001). For example it has been reported that depletion of Rad51C protein in human cells causes a sharply reduction of Xrcc3 protein levels (Lio, 2004. Thus , Xrcc3 may indirectly modulate the HRR process via its interaction with Rad51C. Previously, a modest increment in Rad51C was seen with Xrcc3 overexpression (Wiese et al, 2002).

Moreover it has been show that disruption of Xrcc3-Rad51C and Rad51B-Rad51C interaction using a peptide corresponding to the aminoacids 14–25 of RAD51C sensitized hamster cells to cisplatin and reduced cisplatin-induced Rad51 foci (Connell et al, 2004).

The differences observed in the effect of Xrcc3 expression levels on Rad51 foci formation (occurring during S-phase) and SCEs (post-replicative) may imply that these HRR-related processes involve different sub-pathways in which Xrcc3 plays different roles. In conclusion, our findings have clinical and biological implications. Xrcc3 may be useful as a marker of prognosis in the efficacy of DNA cross-linking agents in the treatment of human tumors. From the biological point of view, the results suggest the existence of cross-talk between HRR and the S-phase checkpoint machinery.

REFERENCES

Aloyz R, Xu ZY, Bello V, Bergeron J, Han FY, Yan Y *et al.* (2002) Regulation of Cisplatin Resistance and Homologous Recombinational Repair by the TFIIH Subunit XPD. *Cancer Res* **62:** 5457-5462.

Aloyz R, Grzywacz K, Xu ZY Loignon M, Alaoui-Jamali MA, Panasci L.(2004) Imatinib sensitized CLL lymphocytes to chlorambucil. *Leukemia* **18**:409-414.

Batist G, Torres-Garcia S, Demuys JM, Greene D, Lehnert S, Rochon M *et al.* (1989) Enhanced DNA cross-link removal: the apparent mechanism of resistance in a clinically relevant melphalan-resistant human breast cancer cell line. *Mol Pharmacol* **36:**224-230.

Bello VE, Aloyz RS, Christodoulopoulos G and Panasci L. (2002) Homologous recombinational repair is-a-vis chlorambucil resistance in chronic lymphocytic leukemia. *Biochem Pharmacol* **63:**1585-1588.

Bishop DK, Ear U, Bhattacharyya A, Calderone C, Beckett M, Weichselbaum RR *et al.* (1998) Xrcc3 is required for assembly of Rad51 complexes in vivo. *J Biol Chem* **273:**21482-21488.

Connell PP, Siddiqui N, Hoffman S, Kuang A, Khatipov EA et al.(2004) A hot spot for RAD51C interactions revealed by a peptide that sensitizes cells to cisplatin. *Cancer Res* **64**:3002-3005.

Christodoulopoulos G, Malapetsa A, Schipper H,Golub E, Radding C and Panasci LC. (1999)

Chlorambucil induction of HsRad51 in B-cell chronic lymphocytic leukemia. *Clin Cancer Res* **5:** 2178-2184.

Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL *et al.* (1998) Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. *EMBO J* **17:**159-169.

De Silva IU, McHugh PJ, Clingen PH, and Hartley JA (2000) Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol Cell Biol* **21**:7980-7990.

Falck J, Petrini JH, Williams BR, Lukas J and Bartel J. (2002) The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. *Nat Genet* **30:** 290-294.

Guo N, Faller DV and Vaziri C. (2002) Carcinogen-induced S-phase arrest is Chk1 mediated and caffeine sensitive. *Cell Growth Differ* **13:**77-86.

Henry-Mowatt J, Jackson D, Masson JY, Johnson PA, Clements PM, Benson FE *et al.* (2003) XRCC3 and Rad51 modulate replication fork progression on damaged vertebrate chromosomes. *Mol Cell* **11:**1109-1117.

Lee SI, Brown MK and Eastman A. (1999) Comparison of the efficacy of 7-hydroxystaurosporine (UCN-01) and other staurosporine analogs to abrogate cisplatin-induced cell cycle arrest in human breast cancer cell lines. *Biochem Pharmacol* **58:**1713-1721.

Lio Y-C, Schild D, Brenneman MA, Redpath JL, and Chen DJ.(2004) Human Rad51C Deficiency Destabilizes XRCC3, Impairs Recombination, and Radiosensitizes S/G2-phase Cells *J Biol Chem* **279**: 42313 – 42320.

Liu N, Lamerdin JE, Tebbs RS, Schild D, Tucker JD, Shen MR, *et al.* (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol Cell* **1:**783-193.

Liu Y, Masson JY, Shaha R, O'Regan P and West S. (2004) RAD51C is required for Holliday junction processing in mammalian cells. *Science* **303:**243-246.

Masson JY, Tarsounas MC, Stasiak AZ, Stasiak A, Shah R, McIlwraith MJ, *et al.* (2001) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev* **15:**296-307.

McHugh PJ, Spanswick VJ and Hartley JA (2001) Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol* **8:**483-490.

Siddik ZH. (2002) Biochemical and molecular mechanisms of cisplatin resistance. *Cancer Treat Res* **112:**263-284.

Slupianek A, Schmutte C, Tombline G, Nieborowski-Skorska M, Hoser G, Nowicki M *et al.* (2001) BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance. *Mol Cell* **8:** 795-806.

Sonoda E, Sasaki MS, Morrison C, Yamaguchi-Iwai Y, Takata M and Takeda S. (1999) Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. *Mol Cell Biol* **19:**5166-5169.

Sonoda E, Takata M, Yamashita YM, Morrison C and Takeda S. (2001) Homologous DNA recombination in vertebrate cells. *Proc Natl Acad Sci* **98:**8388-8394.

Spanswick VJ, Craddock C, Sekhar M, Mahendra P, Shankaranarayana P, Hughes R *et al.* (2002) Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. *Blood* **100:**224-229.

Takata M, Sassaki MS, Tachiiri S, Fukushima T, Sonoda E, Schild D *et al.* (2001) Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. *Mol Cell Biol* **21:**2858-2866.

Tebbs RS, Zhao Y, Tucker JD, Scheerer JB, Siciliano MJ, Hwang M *et al.* (1995) Correction of chromosomal instability and sensitivity to diverse mutagens by a cloned cDNA of the XRCC3 DNA repair gene. *Proc Natl Acad Sci USA* **92:**6354-6358.

Torres-Garcia SJ, Cousineau L, Caplan S and Panasci L. (1989) Correlation of resistance to nitrogen mustards in chronic lymphocytic leukemia with enhanced removal of melphalan-induced DNA cross-links. *Biochem Pharmacol* **38:**3122-3123.

Wang ZM, Chen ZP, Xu ZY, Christodoulopoulos G, Bello V, Mohr G, *et al.* (2001) In vitro evidence for homologous recombinational repair in resistance to melphalan. *J Natl Cancer Inst* **93:**

1473-1478.

West SC (2003) Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol* **435:**435-445.

Wiese C, Collins DW, Albala JS, Thompson LH, Kronenberg A and Schild D (2002) Interactions involving the Rad51 paralogs Rad51C and XRCC3 in human cells. *Nucleic Acid Res* **30:**1001-1008.

Yoshihara T, Ishida M, Kinomura A, Katsura M, Tsuruga T, Tashiro S et al (2004)XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells. *EMBO J* **23**:670-680.

FOOTNOTES

Supported by a NCI (Canada) grant and a generous private donation from the Helen and Nicky Rosenbloom Lang Scholarship to Lawrence Panasci; a U.S. Army grant and a Lymphoma Foundation of Canada fellowship to Raquel Aloyz and a Leukemia Lymphoma Fund of Canada Fellowship to Zhi-Yuan Xu.

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS FOR FIGURES

FIGURE 1:

XRCC3 constitutive overexpression was determined by western blot analysis in 50 μg of protein extracts from **MOCK** cells and **OVER** cells **(A).** The cells were treated with cisplatin or melphalan 24 hours after seeding. Cisplatin (dashed lines) and MLN (solid line) resistance determined by the SRB assay correlated with Xrcc3 protein levels **(B).** The y-axis represent the IC_{50} for the drugs and the x-axis represents Xrcc3 protein levels. The open, grey and black circles represent respectively the results obtained with **MOCK, OVER 1** and **OVER2** cells respectively.

FIGURE 2:

The resistance to CDDP **(A)** and MLN **(B)** was determined using the SRB assay 7 days after treatment. **MOCK** cells (open circles) and two clones of Xrcc3 overexpressing cells **OVER1** (grey circles) and **OVER2** (black circles) cells were treated with cisplatin or melphalan 24 hours after seeding. The IC_{50} values in the tables (μM) represent the mean of 3 independent experiments ± 95% confidence intervals.

The lower panel is a representative survival assay used to obtain by interpolation the IC_{50} values.

FIGURE 3:

MOCK and **OVER** cells were treated with CDDP and stained for Annexin V to assess induction of apoptosis. The y-axis indicates the percentage of Annexin V positive cells at a given time (x-axis) 0-36 hours after 20 μM CDDP treatment. The percentage of **MOCK** and **OVER** cells are represented by open and black bars respectively. The results represent the mean value of 2 independent experiments

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

± 95% confidence intervals. The asterisks *, * and ** indicate significant differences between **MOCK** and **OVER** cells. The respective p values are: 0.042, 0.0016 and 0.045. **(A)**. The lower panel is the analysis of a representative FACS analysis of Annexin V positive cells **(B)**.

FIGURE 4:

Sister cultures of **MOCK** (open) and **OVER** (solid) cells were utilized to determine the effect of Xrcc3 overexpression on HRR as assessed by Rad51 foci determination after cisplatin treatment at 0 μM or 10 μM. Rad51 foci density (**A).** Rad51 density was expressed as **(B)** percentage of control per field (y-axis) vs. time after treatment (x-axis) or **(C)** percentage of control per nucleus (y axis) 12 h after cisplatin treatment. The values represents the mean of 3 independent experiments ± 95% confidence intervals; * and ** indicates significant difference p=0.012 and $0.19x10^{-9}$ respectively.

FIGURE 5:

Sister cultures of **MOCK** (open) and **OVER** (solid) cells were utilized to determine the effect of Xrcc3 overexpression on HRR as assessed by quantification of sister chromatid exchanges (SCEs). SCEs per cell were quantified 36 hour after treatment with 0.5μM or 2.5μM cisplatin (x-axis).The results are expressed as percentage of control (0μM cisplatin, y-axis) (**A**) or per cell (**B**) of the mean value of SCEs per cell determined in 25 cells ± 95% confidence intervals; *, * and ** indicates significant difference p=0.003 and p=0.00043 and $2.6x10^{-6}$ respectively.

FIGURE 6:

Cell cycle progression was determined as described in Material and Methods in sister cultures of **MOCK** (open) and **OVER** (solid) cells. The DNA content of untreated **(left panel)** or treated cells, 6h **(center panel)** and 12hour **(right panel)** after 20 μM cisplatin treatment was determined by FACS analysis after DNA labeling with PI. The values represent the mean of 3 independent experiments ±

JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

95% confidence intervals, *****indicates significant different p=0.024 **(A)**. Representative plots obtained by FACS analysis are reproduced in **B**.

FIGURE 7:

The effect of Xrcc3 overexpression on cisplatin-induced DNA synthesis arrest was determined as described in Material and Methods in sister cultures of **MOCK** (open) and **OVER** (solid) cells. Subconfluent cultures were plated in the presence of ^{14}C-thymidine. Forty-eight hours later, (70-80% confluence) the cells were treated for one hour with 0μM or 10μM cisplatin. The DNA synthesis progression was assessed by a one-hour ^{3}H-thymidine chase (x-axis 0h, 2h, 6h, 12h and 24 hours after treatment). The values represent the mean of 3 independent experiments ± 95% confidence intervals, the asterisk indicate significant differences *****p=0.0096, ** p=0.0074, *** p=0.0012.

FIGURE 8:

The effect of Xrcc3 in signaling proteins involved in cell cycle arrest and apoptosis was determined as described in Materials and Methods in protein extracts of **MOCK** and **OVER** cells by western blot at different times after 20μM CDDP treatment **(A)**. Changes in p53 and p21 protein levels relative to ERK2 and Chk2 phosphorylation status (y-axis) after CDDP treatment (0-48 hour, x-axis) are represented in the right panel **(B)**. The results are a representative experiment of three independent determinations. Cell cycle progression was analyzed 36 hour after treatment as described in Figure VI **(C)**. The profiles are a representative experiment of 2 independent determinations.

FIGURE 9:

Protein extracts from **MOCK** cells (open symbols) and **OVER** cells (solid symbols)were separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed sequentially

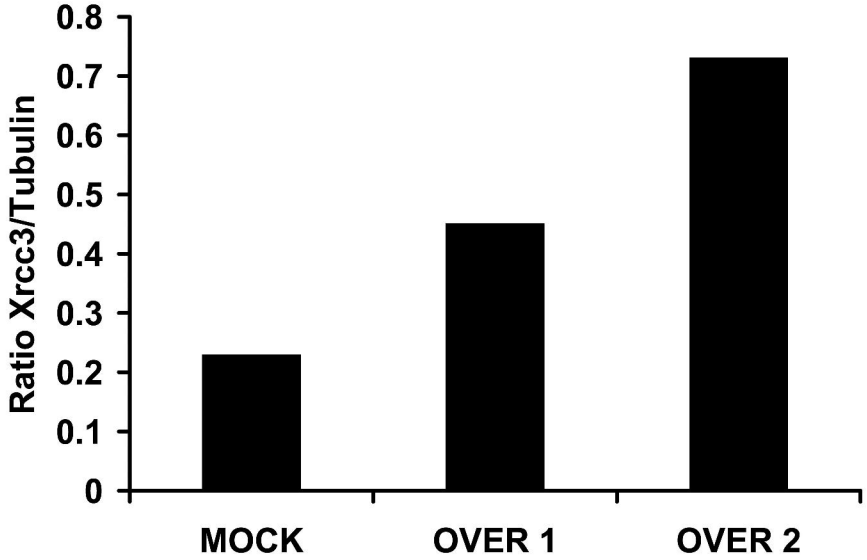
JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

with specific antibodies against Xrcc3, Rad51B and Rad51C (**A left panel)**. The signals in each lane were analyzed using the Scion Image software (**A right panel).** The Xrcc3 optical density (x-axis) correlates with Rad51C optical density (circles) but not with Rad51B optical density (squares). The lineal regression analysis between the optical densities was obtained using the Microsoft Excel Statistical Tool Pack (**B)**. The results indicates that there is a significant linear correlation ($p = 0.036$, $r = 0.902$) between Xrcc3 and Rad51C expression.

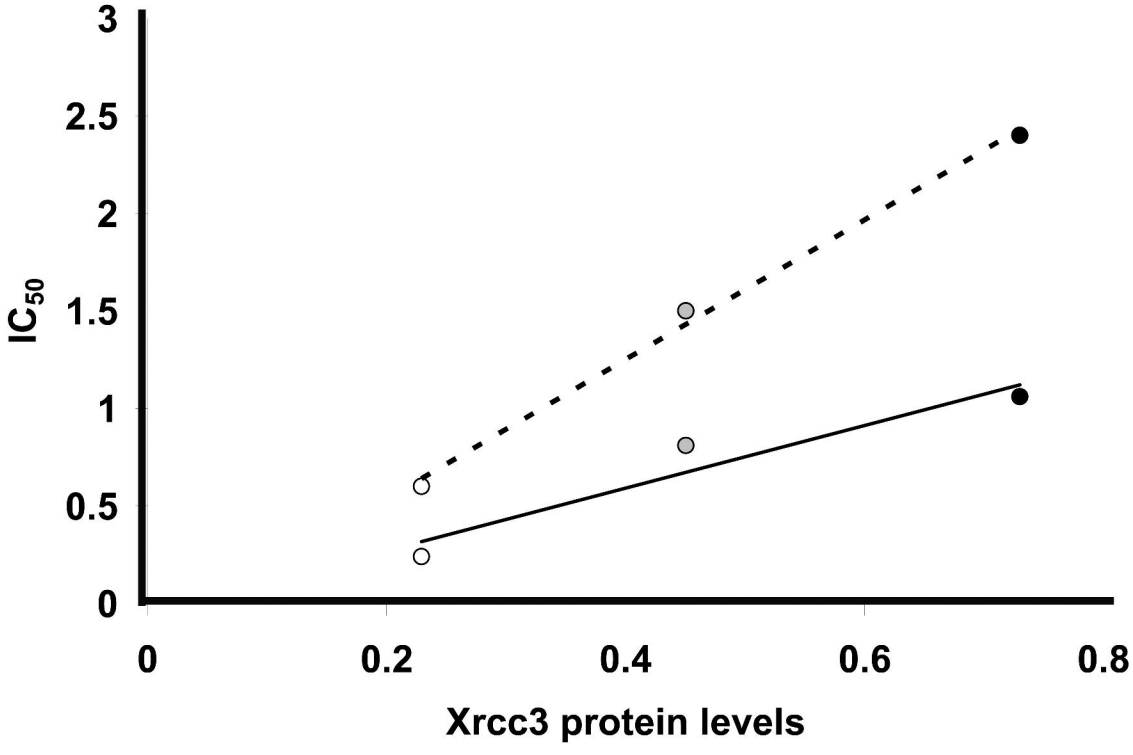
JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

1

A



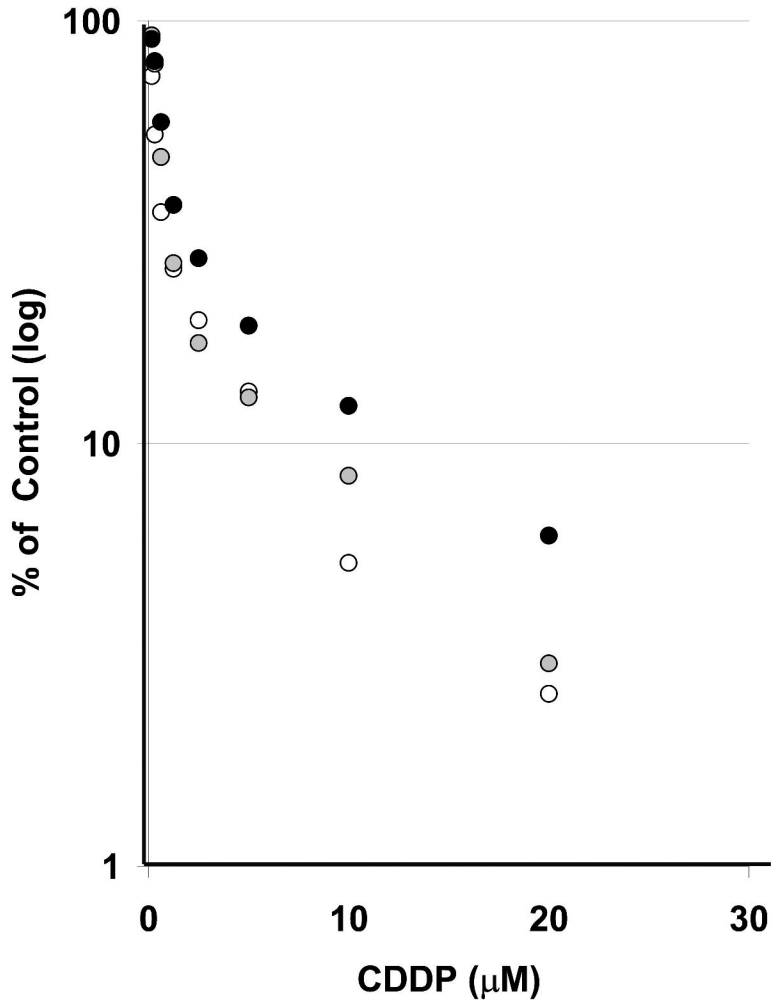
B



JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

2A

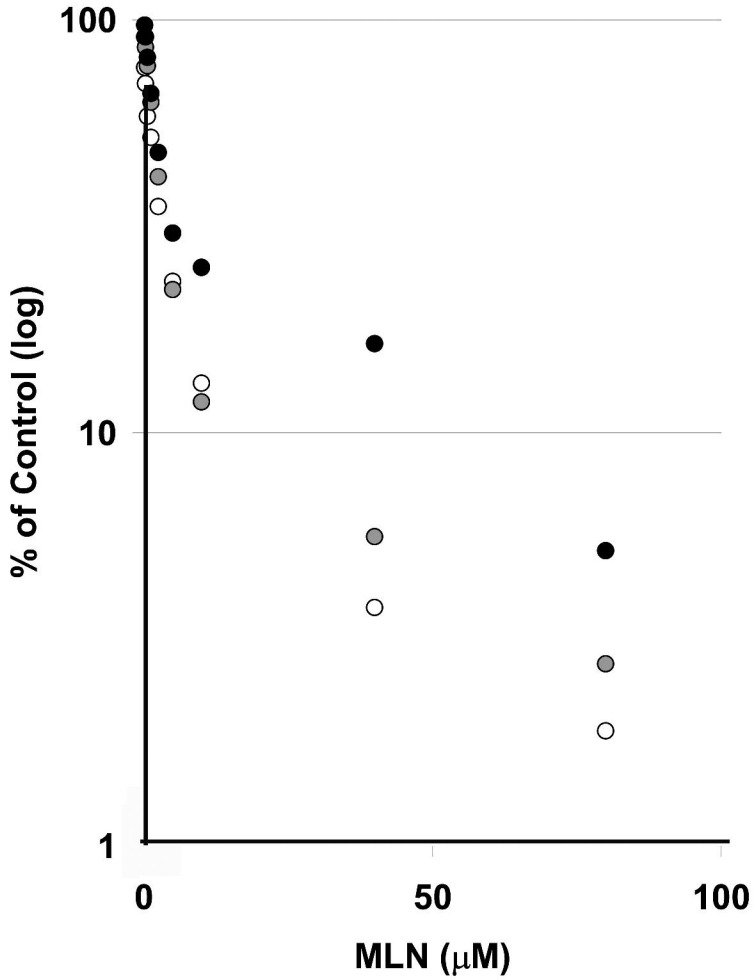
CDDP	IC_{50}	p Value
MOCK	0.24 (0.15to.23)	MOCK vs. OVER1 0.033
OVER 1	0.81 (0.8to0.82	MOCK vs. OVER2 0.016
OVER 2	1.06 (1.05to1.07)	OVER1 vs. OVER2 0.028



JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

2B

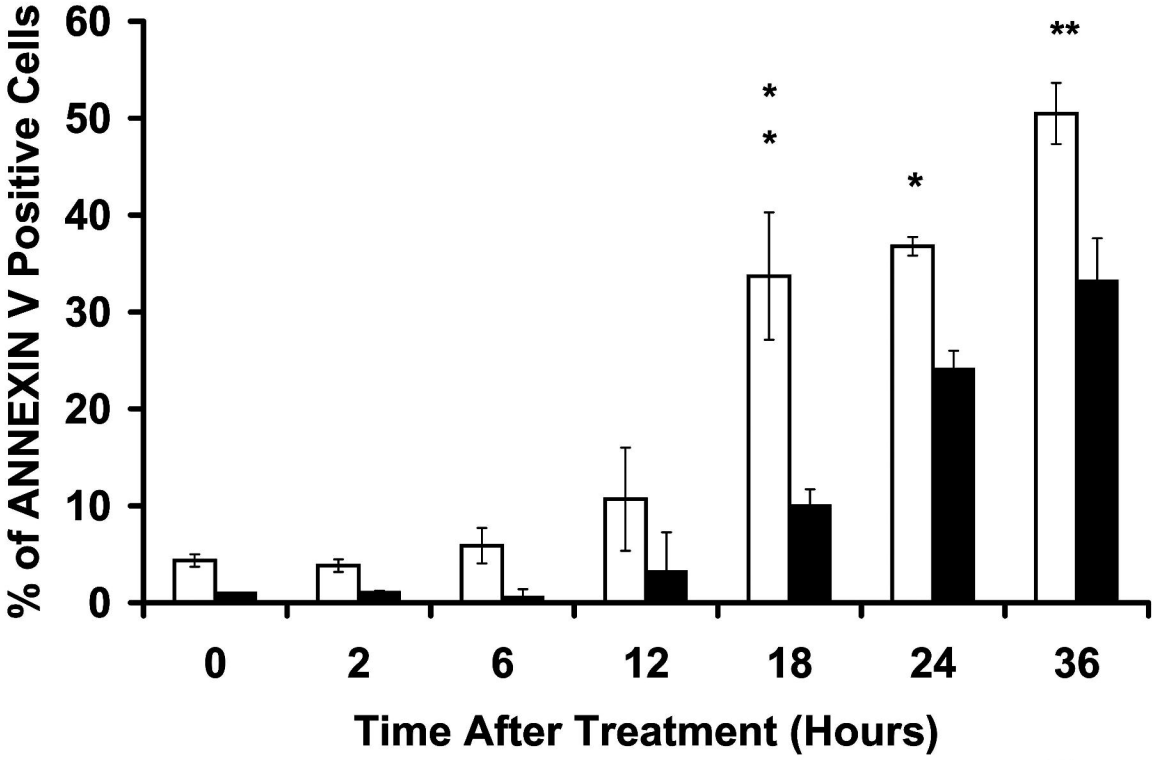
MLN	IC_{50}	p Value
MOCK	0.6 (0.2to1.0)	MOCK vs. OVER1 0.016
OVER 1	1.5 (1.2to 1.8)	MOCK vs. OVER2 0.009
OVER 2	2.4 (1.9to2.8)	OVER1 vs. OVER2 0.028



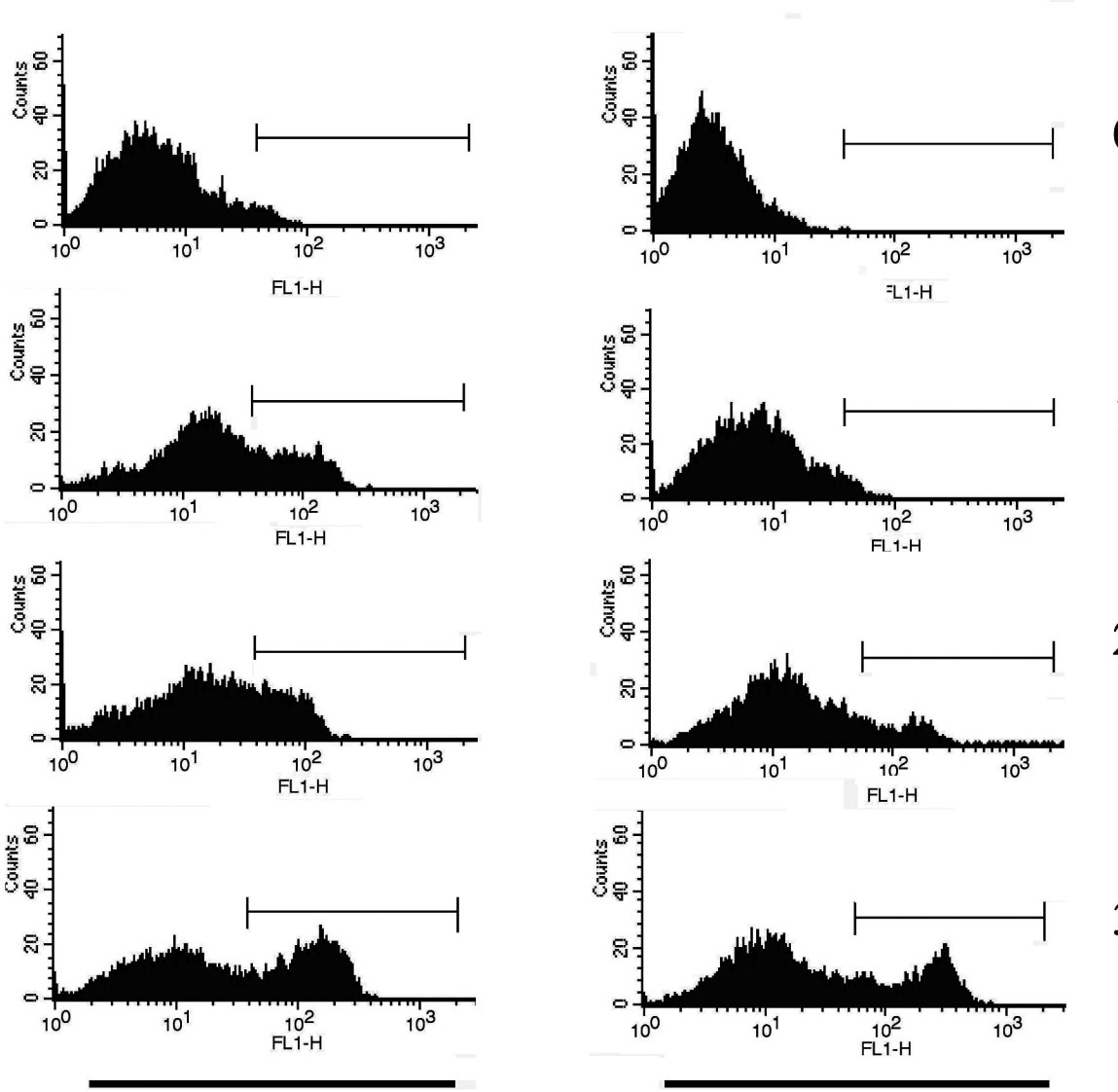
JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

3

A

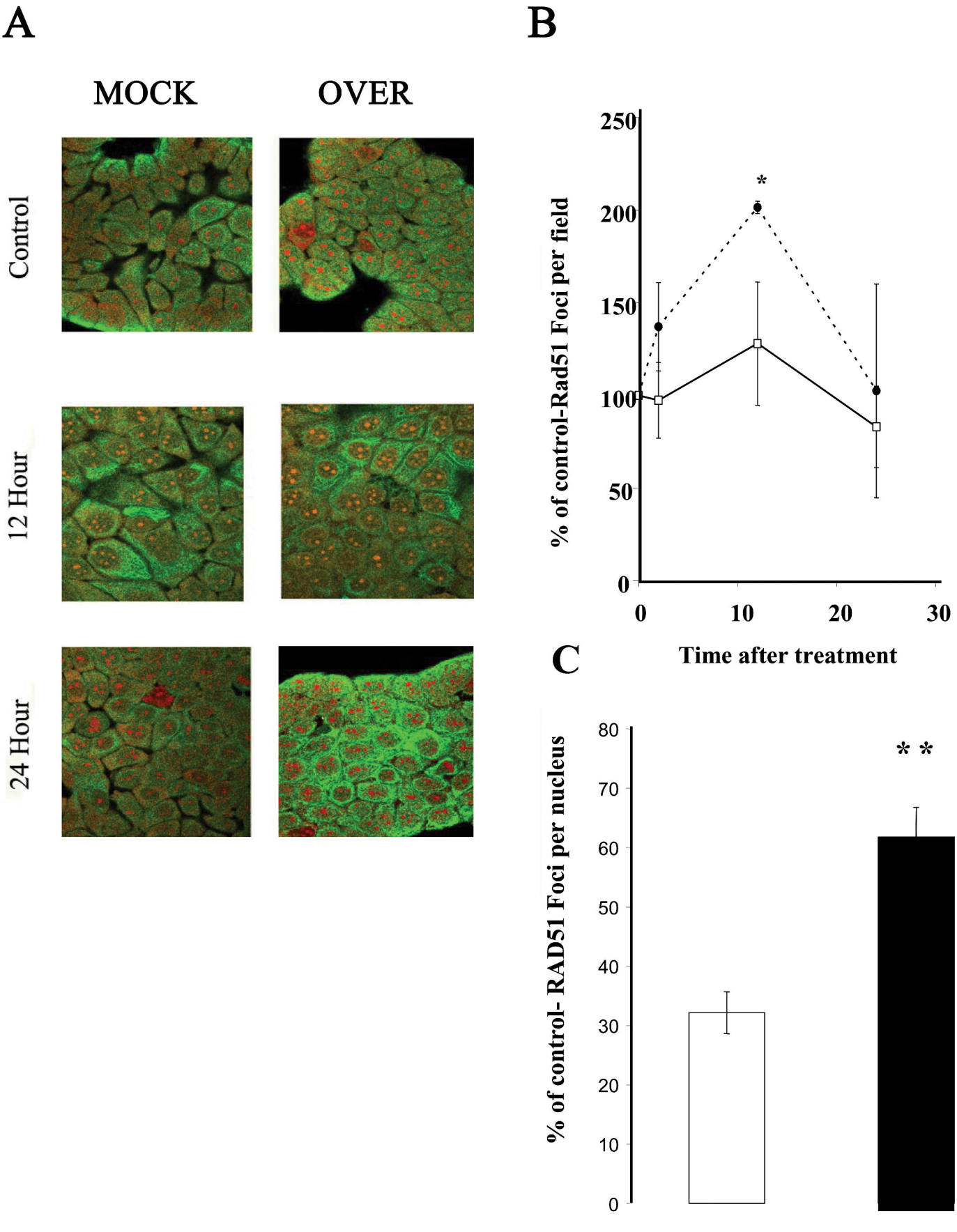


B



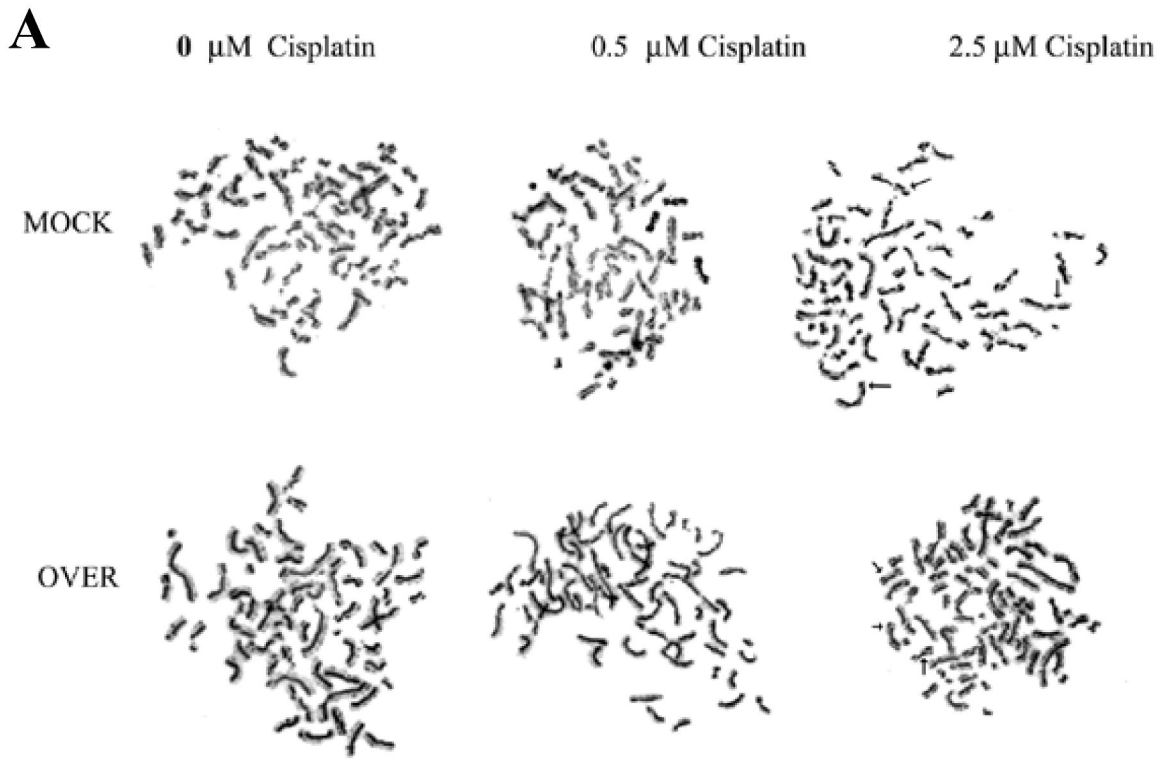
JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

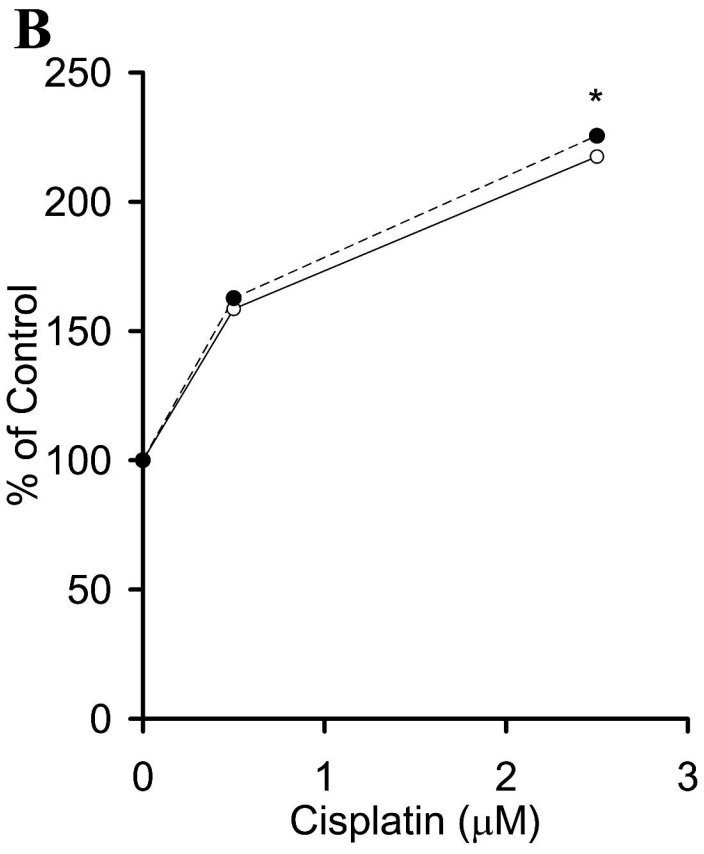
4

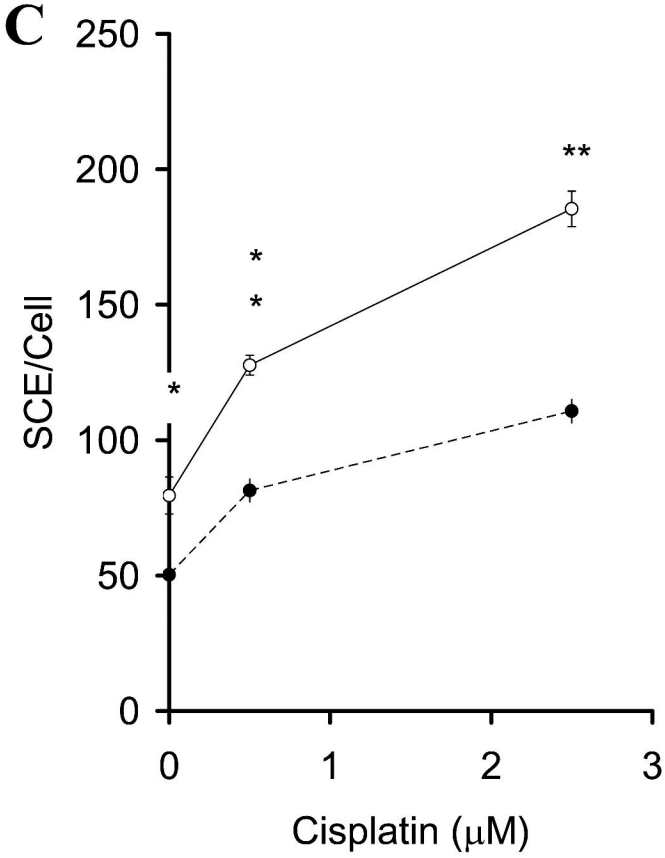


JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

5

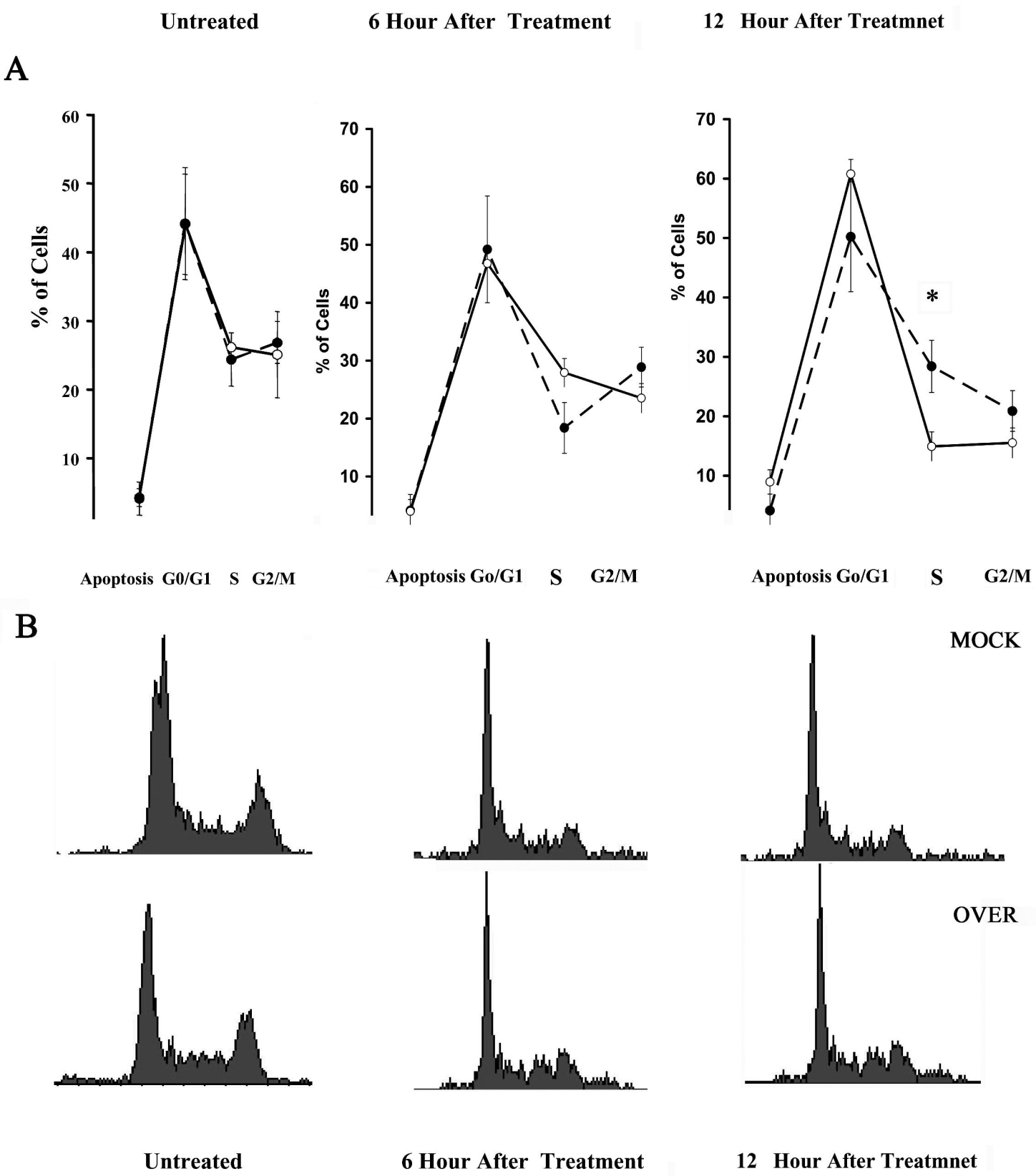






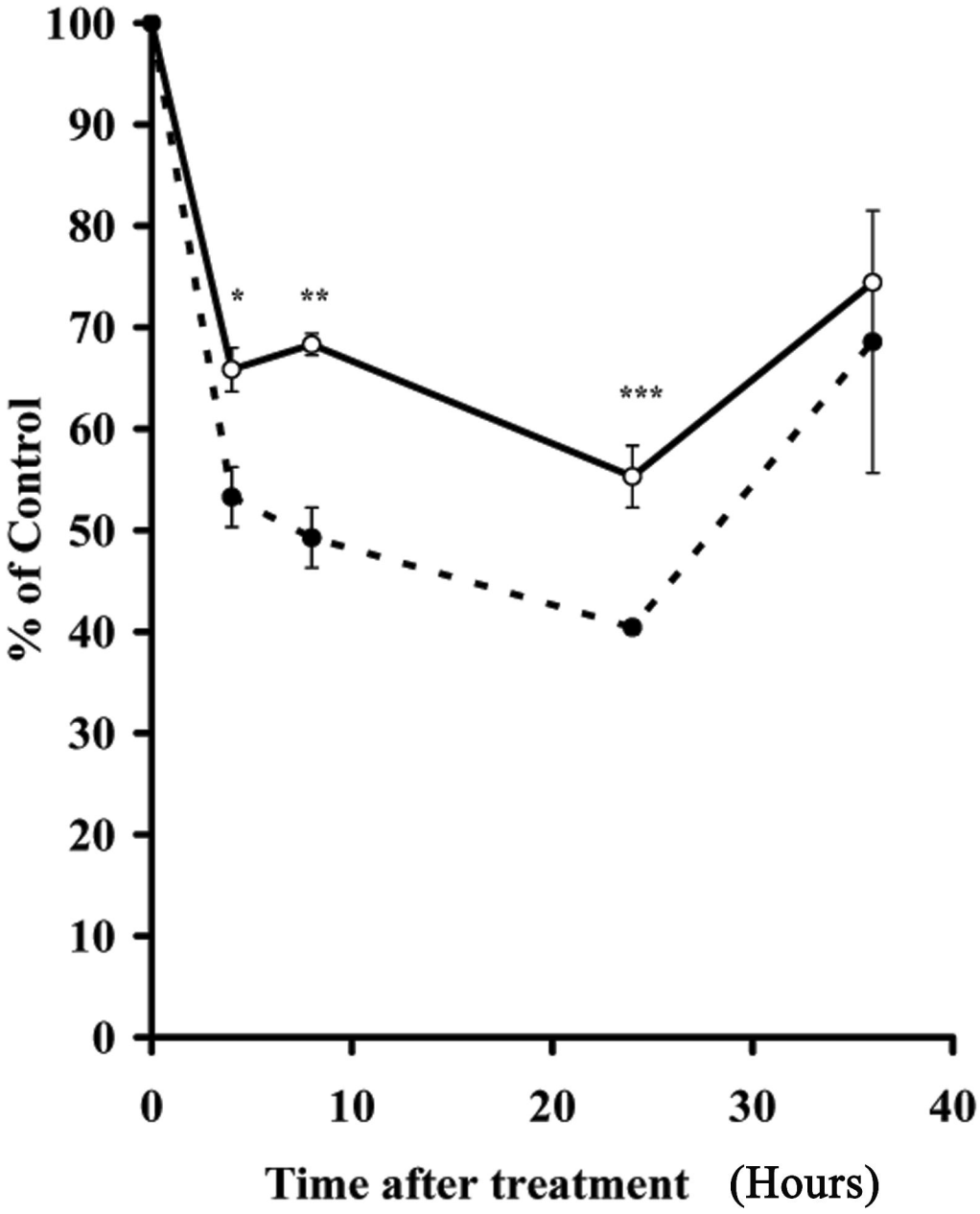
JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

6

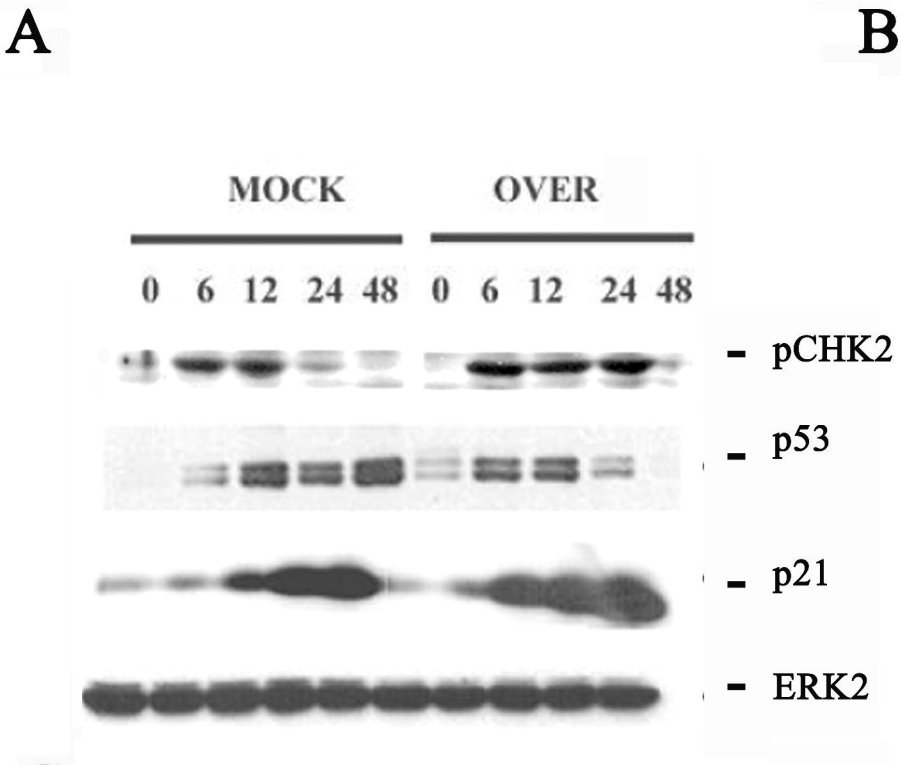


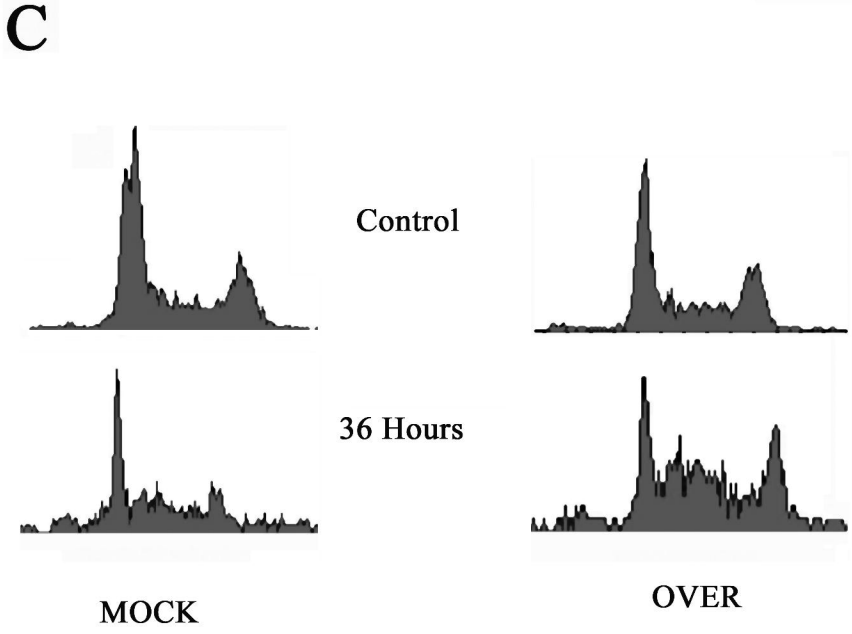
JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

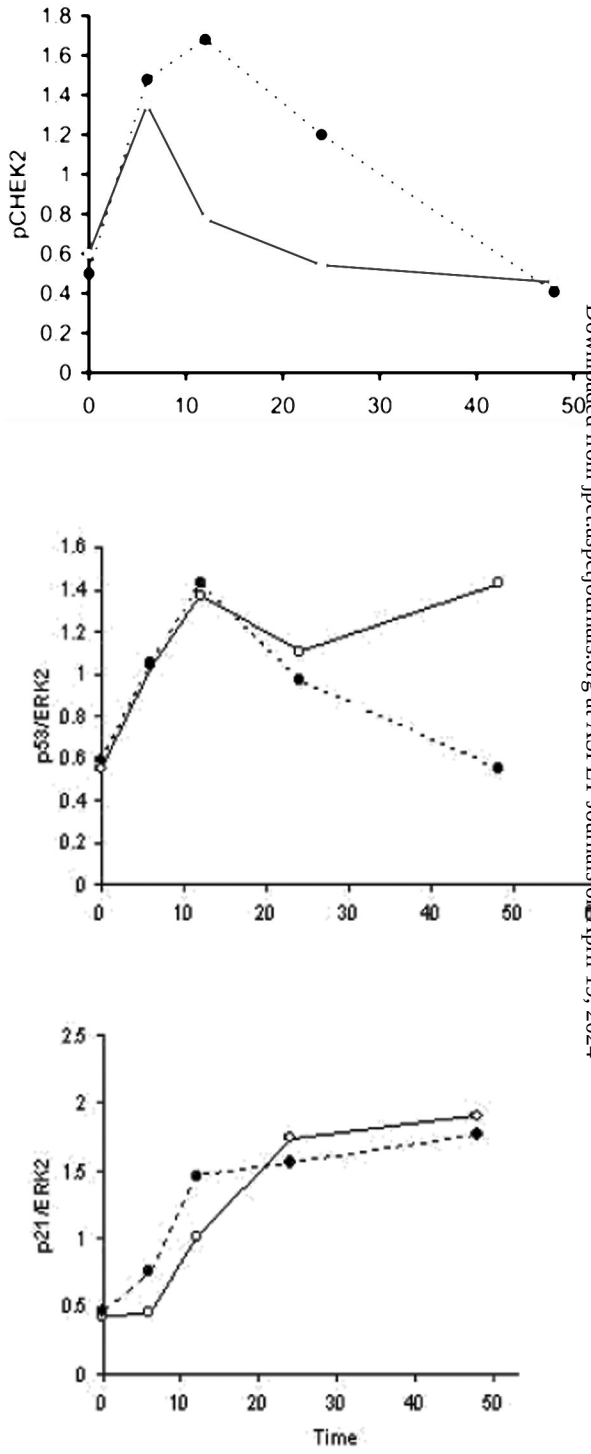
7



JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

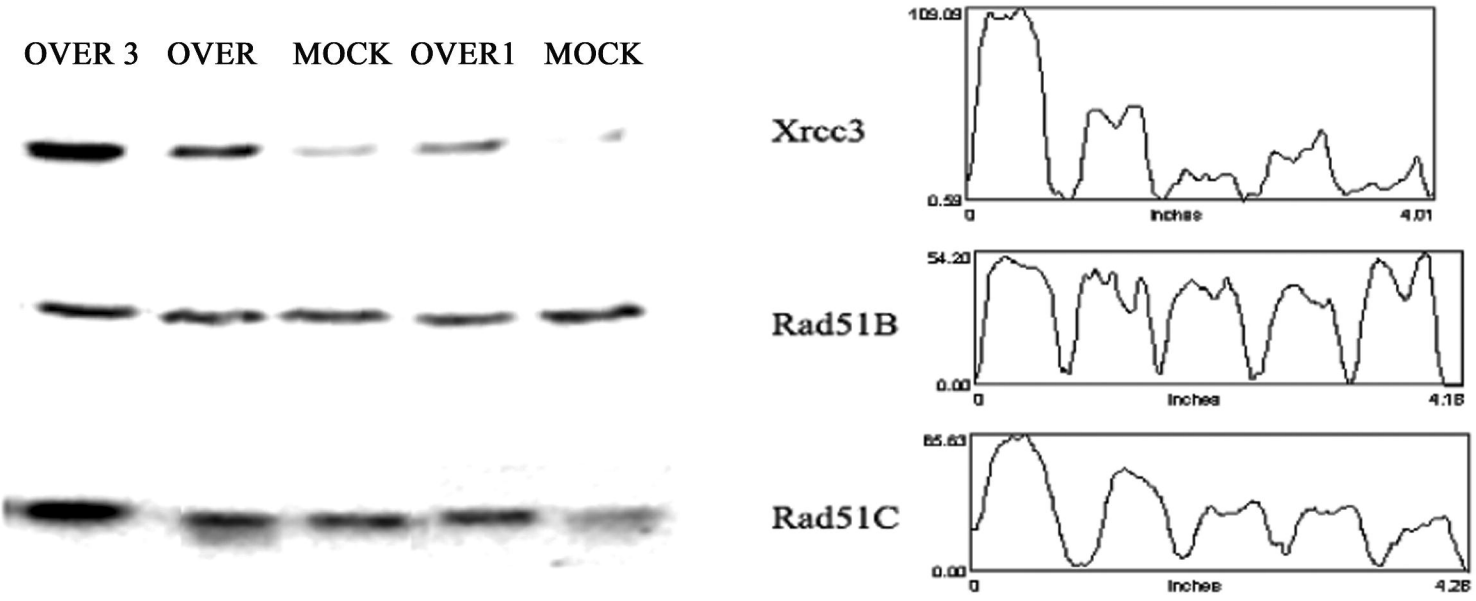






JPET Fast Forward. Published on April 20, 2005 as DOI: 10.1124/jpet.105.084053
This article has not been copyedited and formatted. The final version may differ from this version.

A



B