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Genetic variation of human cytochrome P450 reductase as a potential biomarker for mitomycin C-induced cytotoxicity

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Running title: MMC toxicity and human POR genetic variants

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Non-standard abbreviations

MMC	Mitomycin C
POR	NADPH-cytochrome P450 reductase
CHO	Chinese Hamster Ovary
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl) -2-(4-sulfopheny)-2H-tetrazolium inner salt
IC_{50}	50% Inhibitory Concentration
NQO1	NAD(P)H:quinone oxidoreductase-1

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Abstract

The importance of genetic variation on clinical response to various drugs is now well recognized. Identification of genetic biomarkers that can predict efficacy and toxicity of chemotherapeutic drugs in cancer patients holds a great promise in treatment improvement and cost reduction. Mitomycin C (MMC) is a common anticancer drug used for the treatment of numerous types of tumors. Metabolism-mediated activation, by either one-electron or two-electron reduction, plays a critical role in the chemotherapeutic action of MMC. Cytochrome P450 reductase (POR) is a major enzyme responsible for MMC activation through the one-electron reductive pathway, which leads to the production of semiquinone anion radicals and subsequent DNA damage in the cells. Recently, a total of 6 naturally occurring human POR variants with single amino acid changes (Y181D, A287P, R457H, V492E, C569Y, and V608F) have been identified. Although the catalytic efficiency of these variants in reduction of cytochrome *c* was reported to be altered, their capability in activating MMC, a direct substrate of POR, has not been examined. In the present study, we demonstrated that except for the C569Y variant, MMC-induced toxicity assayed as cell viability and proliferative capability was significantly decreased in the Flp-In CHO cells stably expressing all the other POR variants in comparison with the cells expressing wild-type human POR. Cells expressing the V608F and Y181D variants had a complete loss in the capability to activate MMC. Our finding suggests that these functional POR genetic variations may serve as a potential biomarker to predict the chemotherapeutic response to MMC.

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Mitomycin C (MMC) is a naturally occurring antibiotic that was isolated originally from the microorganism *Streptomyces caspitosus*. It is a highly active anticancer drug commonly used in combination with other chemotherapeutic agents for the treatment of various cancers. It is anticipated that MMC will continue to play its important role in the treatment of bladder, breast, head and neck, and non-small-cell lung cancers (Bradner, 2001). Bioreduction-mediated activation of MMC is responsible for its chemotherapeutic effects. Bioreduction of MMC leads to the formation of free radicals, which causes a cascade of reactions including lipid peroxidation, protein- and DNA-damage, and ultimately cell death (Kappus, 1986). Formation of MMC-induced DNA cross-links has been suggested to be the critical cytotoxic lesion (Hughes et al., 1991). Among the several reductases that were reported to be involved in MMC activation, NADPH-cytochrome P450 reductase (POR), a microsomal flavoprotein, has been demonstrated as a major enzyme (Belcourt et al., 1998; Cummings et al., 1998; Joseph et al., 1996). Human POR is expressed in various normal tissues and a variety of tumor cells (Hall et al., 1989; Yu et al., 2001). It activates MMC through a one-electron reductive mechanism. Reduction of MMC by POR produces semiquinone anion radicals that can be re-oxidized with concomitant formation of superoxide anion radicals. The enzymatic or spontaneous dismutation of superoxide anion radicals can produce hydrogen peroxide and hydroxyl radicals in the presence of trace amounts of iron (Kappus, 1986).

Recently, six genetic variants of human POR, each containing a single amino acid change (Y181D, A287P, R457H, V492E, C569Y, and V608F), have been identified in patients with congential adrental hyperplasia and Antley-Bixler syndrome (Fluck et al., 2004; Arlt et al., 2004). The variant proteins showed a decreased activity in cytochrome *c* reduction *in vitro* (Fluck et al., 2004; Arlt et al., 2004). However, the reported variants were expressed in *E.coli* as truncated proteins (lacking 27 or 46 N-terminal amine acid residues) and their catalytic functions may differ from the full-length POR proteins. It should also be noted that cytochrome *c* is merely an artificial electron acceptor used for the activity assay *in vitro* but not a POR substrate *in vivo*. To further characterize the functional

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significance of these reported POR missense variants, we have recently established Flp-In CHO cell lines that stably express full-length cDNAs of the wild-type or variant human POR and used them to examine the effects of these POR variations on the metabolism and toxicity of paraquat, a direct substrate of POR (Han et al., 2006). However, the effects of these genetic variations on POR-mediated MMC activation remain unknown as the enzymatic activity changes resulted from single amino acid substitutions are often substrate-dependent (He et al., 2004). In the present study, we compared MMC-induced toxicity in these stable transfectant cells and demonstrated that except for the C569Y variant, MMC-induced toxicity was significantly decreased in the cells expressing the other POR variants. This result suggests that cancer patients carrying these variant POR alleles may be less responsive to MMC treatment.

Materials and Methods

Chemicals and reagents. Mitomycin C (>97% purity), crystal violet, NADPH, and cytochrome *c* were obtained from Sigma-Aldrich (St. Louis, MO). Cell Titer 96®AQ$_{uenous}$ non-radioactive cell proliferation assay kit was purchased from Promega (Madison, WI). Flp-In CHO cells, F12 nutrient mixture, fetal bovine serum, penicillin-streptomycin-glutamine, hygromycin B, and trypsin-EDTA were from Invitrogen (Carlsbad, CA).

Flp-In CHO cells expressing human POR. The procedures to establish of the Flp-In CHO cells stably expressing wild-type and variant human POR were described in detail elsewhere (Han et al., 2006). The numbering of the amino acid residues in human POR in this report was based on the NCBI database for the human POR gene (NC_000007), mRNA (NM_000941), and protein (NP_000932). This numbering is consistent with the terminology used by Fluck *et al.* (Fluck et al., 2004) but different from Arlt *et al.* (Arlt et al., 2004) who used the GenBank accession number P16435, which did not include the first three N-terminal amino acid residues (Met-Ile-Asn). Therefore, the Ala284Pro, Arg454His and Cys566Tyr variants reported by Arlt *et al*. were named as Ala287Pro, Arg457His and Cys569Tyr in the report by Fluck *et al*. and in the present study.

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Cell viability determination. Cell viability was determined by observation under a microscope (Nikon ECLIPSE TE2000-S, Japan) and by a modified MTS assay as previously described (Wang et al., 2005). Briefly, the cells were added into a 24-well plate (1×10^5 cells per well) and incubated under 95% humidity and 5% CO_2 at 37ºC overnight. The cells were then treated with MMC (dissolved in 70% ethanol) at different concentrations for 24 hrs. Flp-In CHO cells transfected with pcDNA5 vector alone (containing no POR cDNA insert) were used as a negative control. After MMC treatment, the cells were first subject to microscopic examination. The examination was conducted for all the cells grown in each well of the 24-well plates from at least three separate experiments. The microscopic evaluation was generally performed without knowing the identity of different groups. For MTS assay, the medium in each well was removed and 0.5 ml of MTS mixture was added. The cells were incubated with MTS mixture at 37^{o}C in dark for 30 min. The plate was then read at 490nm wavelength by a μQuant plate reader (Bio-Tek Instrument, Inc., Winooski, VT). In each group, the viability of cells incubated with vehicle only was set at 100%. The IC_{50} values were calculated to compare MMC-induced toxicity among the cells expressing wild-type and different variant POR proteins.

Colony formation assay. The colony-forming ability of cells was determined according to a reported method (Loktionova et al., 1996). The cells were plated into a 6-well plate (5×10^5 cells per well) and grown for 24 hrs. After incubation with MMC (2 μM and 5 μM, determined by the pilot experiment) for 2 hrs, the cells were grown in the replaced fresh medium for 24 hrs. The cells were then replated at a density of 400 cells/100mm dish and grown for 7-8 days until the discrete colonies could be visualized. After washed with phosphate buffered saline, the colonies were stained with 0.5% crystal violet in ethanol and counted. In each group, the cells incubated with vehicle only were set at 100%.

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POR activity assay. POR activity was determined as NADPH-dependent reduction of cytochrome *c* according to a modified protocol (Patterson et al., 1997). Each incubation comprised 100 µl of cytochrome *c* (final concentration 80 µM), 840 µl of reductase assay buffer (50 mM potassium phosphate, 0.1 mM tetrasodium dehydrate, 0.3 M potassium chloride, pH 7.4), and 10 µl of cell lysate (50 µg protein). The reaction was initiated by the addition of 50 µl of NADPH (final concentration 200 µM). The rate of reduction of cytochrome *c* was monitored at 550nm for 3 min against a blank containing the reductase assay buffer only. The activity was expressed as unit per mg protein. One unit of reductase activity is defined as 1 µmole cytochrome *c* reduced per min.

Statistical analysis. The IC_{50} values for cell viability were calculated by a modified logit model. The data were analyzed by SPSS 10.0 for Windows and the differences in cell viability or colony formation capability among groups were determined using one-way ANOVA analysis. A p value of <0.05 was considered statistically significant.

Results

In the present study, Flp-In CHO cells stably expressing wild-type or variant human POR proteins were used to determine the effects of several reported POR genetic variations on MMC-induced cytotoxicity. These cell lines have been recently established in our laboratory (Han et al., 2006). A major advantage of the Flp-In cell system is that all the transfected DNAs are ensured to be integrated, as a single copy, at the same chromosome site (O'Gorman et al., 1991). Thus, it eliminates the variation of transgene expression in different stable transfectant clones as a result of random integration and difference in copy number. This system has been validated and successfully used in our previous studies on different human cytochrome P450 enzymes (Wang et al., 2005; He at al., 2006).

Consistent with many previous studies that demonstrate the important role of POR in MMC bioactivation (Belcourt et al., 1996; Seow et al., 2005), Flp-In CHO cells

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expressing wild-type human POR showed a remarkable increase in MMC-induced toxicity in comparison to the cells transfected with the expression vector alone (containing no POR cDNA). Microscopic examination showed that 24-hr treatment of MMC (20 μM) in the cells expressing wild-type POR caused abnormal changes in cell morphology and a significant reduction in the number of cells (Fig. 1). Cells expressing the C569Y variant responded to the MMC treatment similarly as the wild-type POR cells. In contrast, cells expressing the other human POR variants were more resistant to MMC-induced toxicity than the cells expressing wild-type POR (Fig. 1). In particular, the response to MMC in the cells expressing the Y181D and V608F variants was very similar to that in the cells transfected with the vector alone (Fig. 1). The alterations of MMC-induced toxicity in the cells expressing different POR variants were further confirmed by MTS assay which measures the mitochondrial dehydrogenase released during cell death. The Flp-In CHO cells expressing wild-type human POR and the C569Y variant were much more sensitive to MMC than the vector control cells (Fig. 2). The IC_{50} (μM) of MMC in the cells expressing wild-type POR and the C569Y variant were 2.5 and 3.1, respectively; while the value for the vector control cells was 17.1. Cells expressing the Y181D and V608F variants showed similar response to MMC as the vector control cells with IC_{50} values of 14.1 μM and 16.9 μM, respectively. Cells expressing the A287P, R457H and V492E variants, while were still more sensitive to MMC than the vector control cells, also showed an increased viability in comparison with the cells expressing wild-type POR (Fig. 2).

In addition to causing cell death, MMC has a potent antiproliferative effect (Granada et al., 2005). We, therefore, also used colony formation assay to compare the effect of MMC on the proliferative capability in the cells expressing wild-type human POR or its variants. Our pilot experiment showed that the plating efficiency of the Flp-In CHO cells without MMC treatment was approximately 50-65%. MMC treatment at 1 μM for 24 hrs or 10 μM for 2 hrs resulted in a complete loss in the ability of colony formation for all the cells, including the vector control cells and the cells expressing either wild-type POR or

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the variants. Therefore, we selected the treatment protocol of 2 μM and 5 μM MMC for 2 hrs. The result of colony formation assay showed the same trend as seen in the cell viability study. Except for the C569Y variant, cells expressing the other human POR variants showed an increase in cell proliferating capability in comparison with the cells expressing wild-type POR. Again, cells expressing the Y181D and V608F variants showed the same response to MMC as the vector control cells (Fig. 3).

As shown in Fig. 4, POR activity in these stable transfectant cells correlated very well with both the cell viability (Fig. 2) and colony formation data (Fig. 3). While there was little endogenous POR activity in the cells transfected with vector alone, the activity was increased approximately 30 folds in the cells expressing wild-type human POR. Except for the C569Y variant, cells transfected with all the other POR variant cDNAs showed a significant decrease in POR activity in comparison with the cells expressing wild-type POR (Table 1).

Discussion

All our results provide direct evidence that the POR genetic variations (except for C569Y) examined in the present study have a significant impact on POR-mediated cytotoxicity of MMC. The molecular mechanisms involved in the POR activity alteration, however, could be different. Our recent work with immunoblot and RT-PCR analyses suggests that while protein structure alteration is responsible for the activity changes in the R457H, V492E and V608F variants, a decrease in protein and/or mRNA stability is probably the major mechanism for the activity changes in the A287P and Y181D variants (Han et al., 2006). The lack of activity change in MMC activation in the cells expressing the C569Y variant is consistent with our recent finding that these cells have the same sensitivity as the cells expressing wild-type POR to paraquat-induced cell death (Han et al., 2006). In contrast, the expressed C569Y variant protein was reported to show an activity decrease in cytochrome *c* reduction in two previous studies (Fluck et al., 2004; Arlt et al., 2004). The discrepancy is probably due to the use of truncated POR proteins in their studies. It has been observed that the N-terminal sequence of POR is important for the electron

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transfer function (Black et al., 1979; Bonina et al., 2005). Although the study by Balck et al. (1979) suggested that the membrane anchor of POR is required for interaction and reduction of cytochrome P450 but not cytochrome *c*, it is not clear whether the reported activity decrease in cytochrome c reduction could be due to a combined effect of C569Y change and the N-terminal truncation in the POR protein.

In addition to human POR, NAD(P)H:quinone oxidoreductase-1 (NQO1) has been reported to be another major enzyme for MMC activation through two-electron reduction (Cummings et al., 1998). A 609C>T (P187S) polymorphism of NQO1 (NQO1*2), which results in a significant reduction in enzyme activity, was reported to be associated with poor survival in patients with disseminated peritoneal cancer receiving intraperitoneal hyperthermic MMC (Fleming et al., 2002) and in non-small cell lung cancer patients treated with MMC-based chemotherapy at relapse (Kolesas et al., 2002). It is, therefore, reasonable to hypothesize that functional genetic variations of POR, such as the ones identified in the present study, may also significantly influence the therapeutical effects of MMC. Further studies are warranted to determine the therapeutical response to MMC in cancer patients carrying these functional POR variant alleles, either alone or in combination with the functional NQO1 variant alleles.

A significant increase in sensitivity to MMC was observed in CHO cells expressing human POR under both aerobic and hypoxic conditions, although the cytotoxicity was greater under latter conditions (Belcourt et al., 1996; 1998). In contrast, POR-mediated activation of adriamycin, another quinone anticancer drug, occurs only under aerobic conditions (Bartoszek, 2002). It should be noted that the present MMC cytotoxicity study was conducted under aerobic condition. It is possible that under hypoxic condition the difference in MMC toxicity between the wild-type POR cells and variant POR cells might be even larger. This prediction needs to be examined by future experiments.
In addition to MMC, POR is directly involved in the bioactivation of other quinone anticancer drugs (Bachur et al., 1979; Rooseboom et al., 2004). By supporting the

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catalytic function of microsomal cytochrome P450 enzymes, POR also plays an indirect yet critical role in the metabolism of chemotherapeutic drugs that are substrates of cytochrome P450 enzymes such as cyclophorsphamide (Chen et al., 1997) . It would be of great interest to determine if the clinical response to these non-MMC anticancer drugs is affected by genetic variations of human POR.

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Footnotes

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Legends for figures

FIG. 1. *MMC-induced changes in confluency and morphology in Flp-In CHO cells expressing wild-type and variant human POR.* Except for the control group, all the cells (including the vector group) were treated with 20 μM MMC for 24 hrs.

FIG. 2. *Effects of MMC on viability in Flp-In CHO cells expressing wild-type and variant human POR.* Cells were treated with different concentrations of MMC for 24 hrs, and the cell viability was determined by MTS assay. The viability in non-treated cells was set at 100%. The results are mean ± SD from triplicate samples. ◇ vector, ◆ wt, Δ Y181D, ▲ A287P, ○ R457H, ● V492E, □ C569Y, ■ V608F. "#", $P<0.05$ in comparison with the vector control cells; "*", $P<0.05$ in comparison with the cells expressing wild-type POR.

FIG. 3. *Effects of MMC on colony formation capability in Flp-In CHO cells expressing wild-type and variant human POR*. Cells were treated with 2 and 5 μM of MMC for 2 hrs. The colony formation capability of the cells without MMC treatment was set at 100%. The results are mean ± SD from triplicate samples. ◇ vector, ◆ wt, Δ Y181D, ▲ A287P, ○ R457H, ● V492E, □ C569Y, ■ V608F. "#", $P<0.05$ in comparison with the vector control cells; "*", $P<0.05$ in comparison with the cells expressing wild-type POR.

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Table 1

POR activity in Flp-In CHO cells expressing wild-type and variant POR

Cells	Cytochrome *c* reduction activity
Vector	6.26±1.59 *
WT	172.10±16.80 #
Y181D	7.97±2.39 *
A287P	50.86±10.82 #,*
R457H	75.68±18.76 #,*
V492E	26.89±5.45 #,*
C569Y	202.99±25.58 #
V608F	16.30±3.88 *

The activities were expressed as units/mg.protein. Cytochrome *c* reduction assay was conducted with cell lyslate proteins and the values are mean ± SD of three separate experiments. "#", $P<0.05$ in comparison with the vector control cells; "*", $P<0.05$ in comparison with the cells expressing wild-type POR.

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Fig. 1

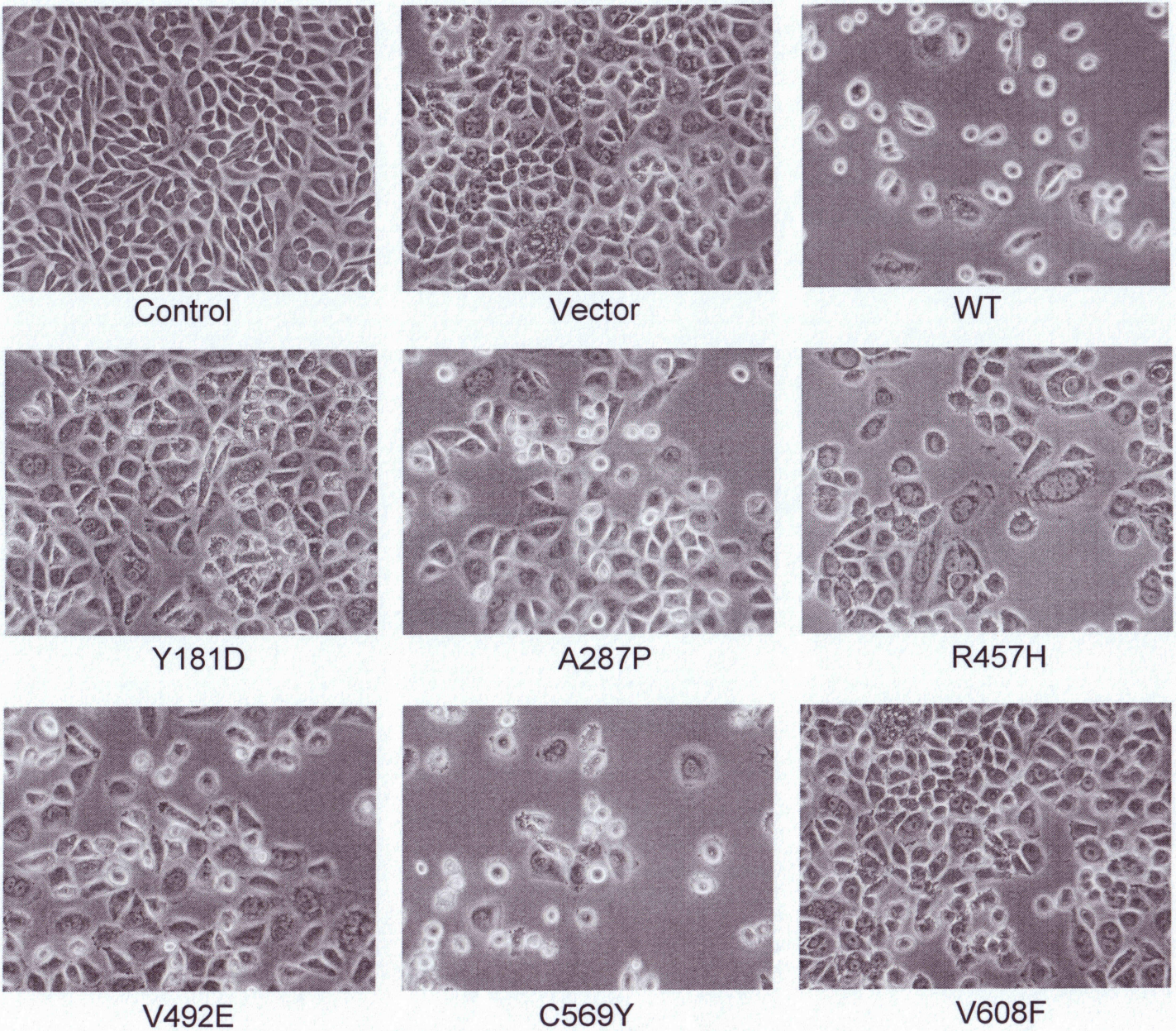

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Fig. 2

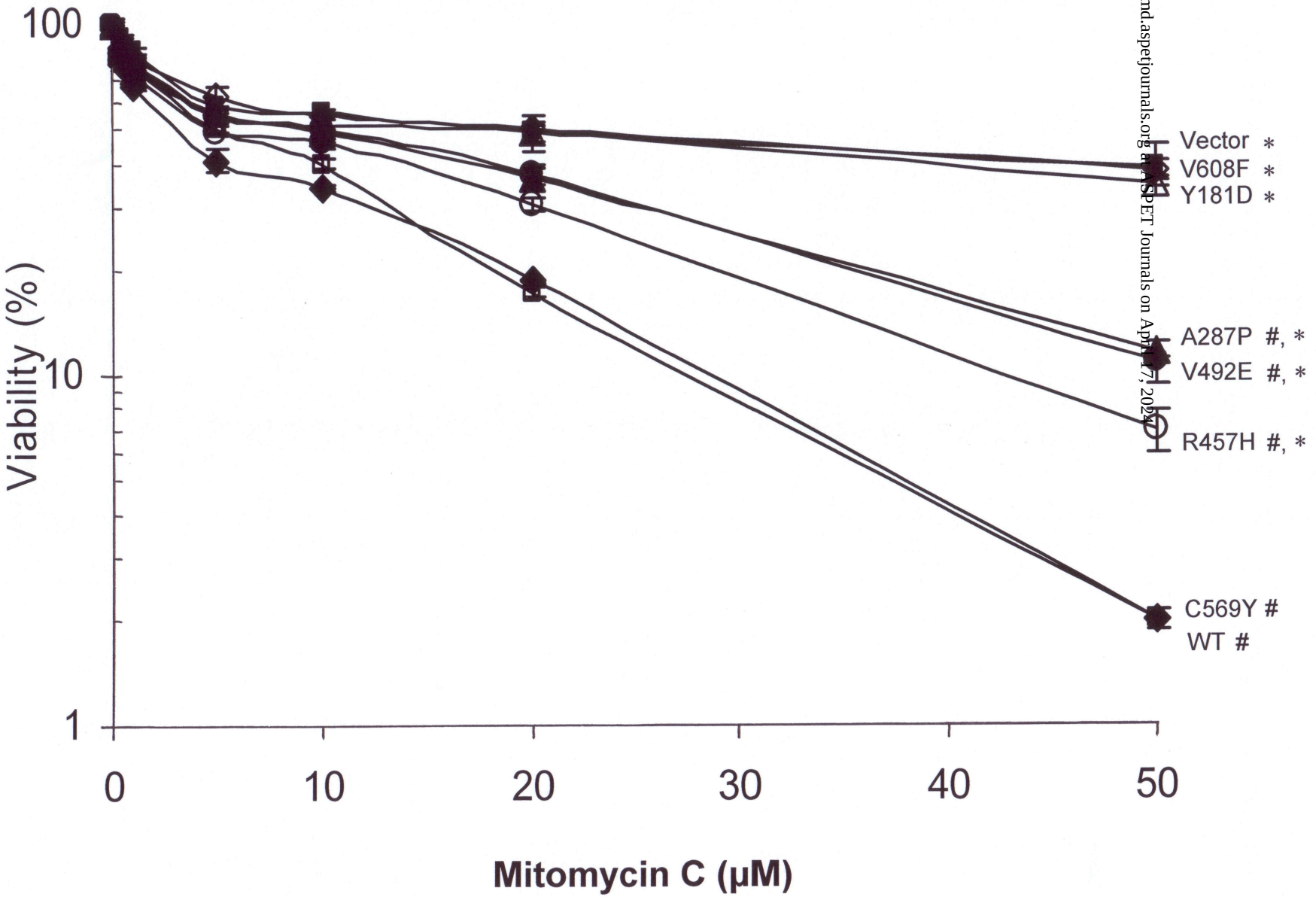

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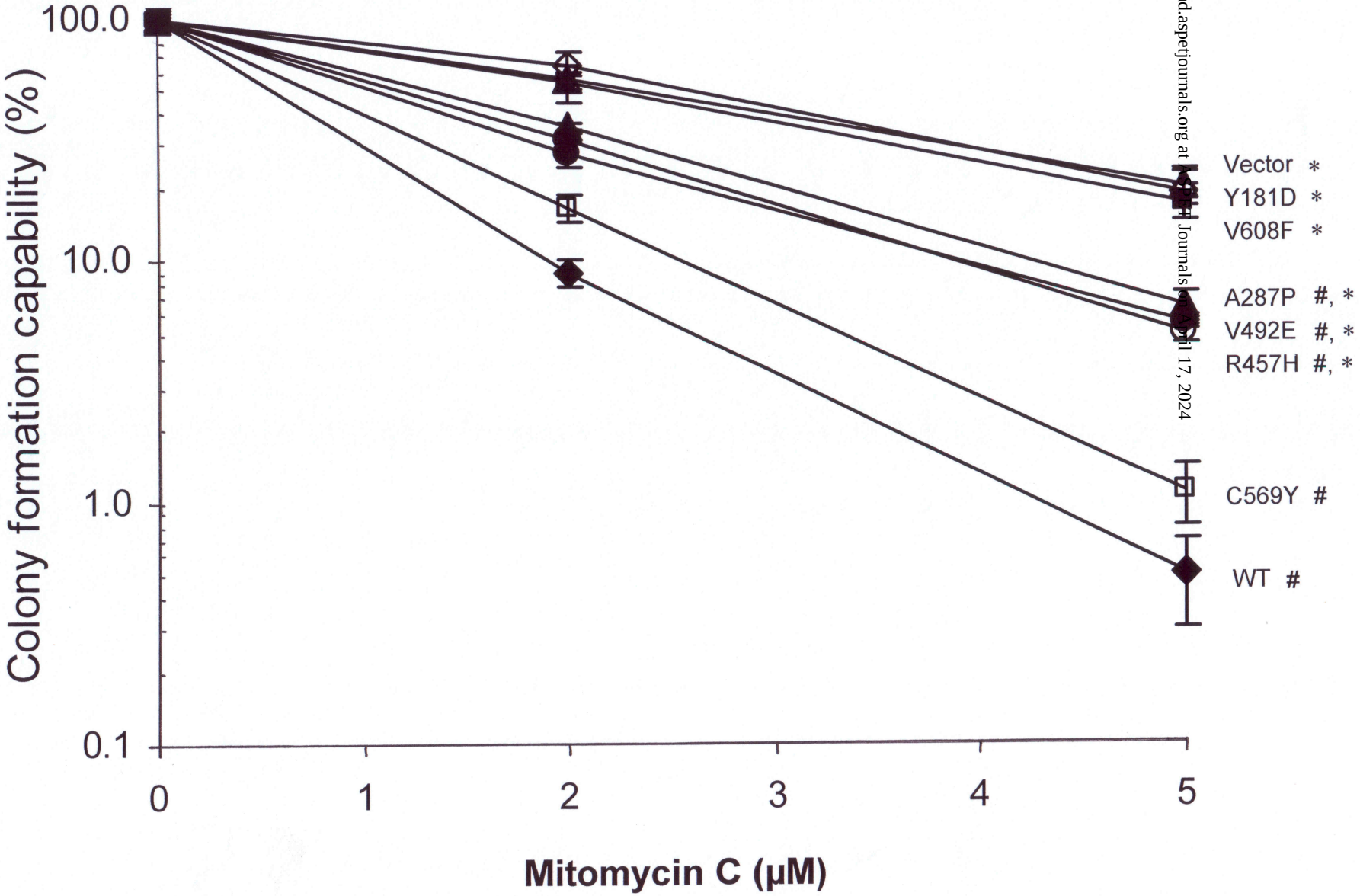
Fig. 3



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