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Effects of the Mortalin Inhibitor MKT-077 on the Tumor Suppressor p53 in Neuroblastoma IMR-32 Cells

Senior Honors Thesis
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Abstract – The tumor suppressor protein, p53, is an important cell cycle regulator in humans. Over half of all human cancers involve disruption of p53 function. One way this is achieved is by tethering p53 to the mitochondrial 70 kilodalton heat shock protein (Hsp70), mortalin, in the cytoplasm, and preventing p53 from entering the nucleus. The mortalin inhibitor, MKT-077, binds competitively to the p53 binding site in mortalin, and disrupts the p53-mortalin complex in cancer cell lines, allowing p53 to enter the nucleus and promote apoptotic cell death. Previous research reported that cytoplasmic tethering of p53 occurs in certain human neuroblastomas. Thus, we have studied the effects of MKT-077 on the human neuroblastoma cell line, IMR-32, using viability assays to determine cell death following treatment with MKT-077 (0 µM to 10 µM), and using immunocytochemistry to localize p53 within the cell. There was a positive correlation between cell death and concentration of MKT-077. Treatment with MKT-077 increased cell death from 21% to 78% as the concentration increased from 2 µM to 10 µM. Immunocytochemistry showed that p53 was located in the cytoplasm in untreated cells, and that treatment with MKT-077 caused it to enter the nucleus, and to become more concentrated there at higher concentrations of MKT-077. Overall, the results suggested that the p53-mortalin complex is likely to be present in IMR-32 cells, and that mortalin inhibitors could be a group of agents that are effective in selectively targeting cells characterized by this complex.

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Background

Summary – The tumor suppressor p53 is present in all human cells and prevents cancer formation by protecting the integrity of the genome. It is capable of inducing apoptosis in cells with highly mutated DNA. It is a highly central protein in cancer biology; over half of all human cancers involve the disruption of p53. It generally acts as a transcription factor and thus needs to enter the nucleus in order to induce apoptosis (Ozaki and Nakagawara, 2011). Cancers can bypass p53 through the over-expression of the mitochondrial matrix protein, mortalin, which can bind to p53 in the cytoplasm, sequestering it there and preventing it from causing apoptosis. Consequently, the cancer proliferates. This p53-mortalin complex has been identified in a number of cancer cell lines (Walker et al., 2006). In cell lines where the complex is present, mortalin inhibitors, such as the chemical agent MKT-077, have been effective in freeing p53 by competitively binding to mortalin, thus allowing p53 to enter the nucleus and trigger apoptosis (Wadhwa et al., 2000). The purpose of the current study was to investigate the presence of the p53-mortalin complex and the effects of MKT-077 in the human neuroblastoma cell line IMR-32.

Tumor Suppressor p53 – The tumor suppressor protein, p53, has been referred to as the “guardian of the genome” for its role in preventing cancer by protecting the integrity of cellular DNA. It is present in low concentrations in the cytoplasm of all human cells, and it is activated (generally via phosphorylation) during the G1/S growth checkpoint of the cell cycle. This is the point at which the cell is checking that its DNA is undamaged,
immediately prior to replicating it in preparation for mitosis. At this point, other proteins are involved in detecting mutations in the DNA—mutations that could disrupt the cell cycle and therefore lead to tumor formation—and inducing p53 to act accordingly (Ozaki and Nakagawara, 2011).

The action of p53 is generally as a transcription factor. It enters the nucleus, forms a tetramer around the DNA, and induces the transcription of appropriate genes depending on the severity of mutations detected. At mild levels of mutation, p53 induces the transcription of DNA editing and repair proteins to fix the mutations. At moderate levels of mutation, p53 induces the transcription of proteins that halt the cell cycle and arrest the growth of the cell, thereby preventing the mutations from spreading. Finally, at irreparable levels of mutation, p53 induces the transcription of the pro-apoptotic protein BAX, which will lead to the death of the cell via apoptosis (Ozaki and Nakagawara, 2011).

While the current study focuses on the “traditional” transcriptional pathways of p53—which require its entry into the nucleus—it is important to note that there are other p53 pathways that don’t require entry into the nucleus. For example, there is a mitochondrial pathway in which p53, in response to stress, directly interacts with and inhibits the anti-apoptotic mitochondrial outer membrane protein Bcl-2. This leads to the permeabilization of the mitochondrial outer membrane, which initiates apoptosis by releasing numerous apoptotic proteins into the cytoplasm (Vaseva and Moll, 2009).
p53-Mortalin Complex – For a tumor to form, it must first find a way to bypass the tumor suppressant function of p53. One way this happens is by the action of the mitochondrial matrix chaperone protein, mortalin. Mortalin, also known as GRP-75 or mot-2, is a 70-kilodalton heat shock protein (Hsp70). Mortalin binds p53, and by binding to wild-type p53 in the cytoplasm—which is constitutively present in low concentrations—it sequesters p53 there and prevents its entry into the nucleus. This will generally prevent p53-induced apoptosis, and consequently, other mutations disrupting the cell cycle can then allow a tumor to form (Walker et al., 2006).

Mortalin is an endogenous protein, which is generally present at low enough concentrations that its tendency to bind to some of the p53 in cells is not an issue. However, in the case that mortalin becomes over-expressed due to a mutation, the p53-mortalin complex can become prevalent in cells. As such, the p53-mortalin complex is thought to contribute to the development of cancer. In fact, this complex has been identified in cell lines belonging to a wide variety of cancers, including but not limited to: undifferentiated neuroblastoma, retinoblastoma, hepatocellular carcinoma, colorectal carcinoma, glioblastoma (Walker et al., 2006), breast carcinoma, osteosarcoma, and fibrosarcoma (Grover et al., 2012).

Mortalin Inhibitor MKT-077 – The rhodacyanine dye, MKT-077, is a chemical agent capable of competitively inhibiting mortalin by binding to a site close to where p53 binds. In other human cancer cells lines where MKT-077 was tested, it successfully freed p53
from sequestration in this way, allowed it to enter the nucleus and trigger apoptosis, and caused the death of the cancer cells. Targeting the p53-mortalin complex in this way is ideal because it makes it possible for a chemical agent to be selectively toxic to cancer cells. The p53-mortalin complex, when present in a cancer, is a major contributing factor to the cancer’s development, and would therefore not be present in healthy cells (Wadhwa et al., 2000).

It is important to note that MKT-077 in particular has been shown to exhibit selective anti-cancer cytotoxicity through other pathways as well, beyond the disruption of the p53-mortalin complex. In particular, MKT-077 can accumulate in the mitochondria of cancer cells and heavily damage them, leading to cell death. This is a necrotic mechanism—in contrast to MKT-077’s disruption of the p53-mortalin complex, which is apoptotic (Modica-Napolitano et al., 1996).

**Neuroblastoma** – Neuroblastoma is a cancer that develops from neuroblasts (immature nerve cells) of the neural crest. It is one of the most common childhood cancers—particularly in early childhood—being the most common cancer in infants under one year of age (Birch and Blair, 1992). Neuroblastomas can be classified according to the level of differentiation of the cancerous neuroblasts (Moll et al., 1995).

**IMR-32** – IMR-32 is a human neuroblastoma cell line that will be used for the current study. It is an adherent cell line that was derived in April 1967 from a 13-month old boy
who had neuroblastoma. The neuroblastoma was undifferentiated, but was described to have “rare areas of organoid differentiation.” IMR-32 cells were derived from the metastatic site (an abdominal mass), grown in culture, and submitted to the American Type Culture Collection (ATCC) in the 36th passage. The IMR-32 cell population is actually comprised of two distinct cell types which are thought to share a common origin—one of the cell types is characterized by a fibroblastic morphology, and the other is round (Tumilowicz et al., 1970).

**Experiment and Rationale** – In the current study, IMR-32 cells were treated with various concentrations of MKT-077, and its effect on the localization of p53 within the cell, as well as its ability to cause IMR-32 cell death, were determined. The p53-mortalin complex in particular has not been studied in IMR-32 cells or in neuroblastoma in general, but cytoplasmic sequestration of p53 has been shown to be very common in undifferentiated (but not differentiated) neuroblastomas (Moll et al., 1995). The purpose of the current study, therefore, was to investigate whether p53 is cytoplasmically sequestered in IMR-32 cells, whether the p53-mortalin complex is responsible for this sequestration (if present), and whether MKT-077 would be effective in disrupting the p53-mortalin complex in IMR-32 cells (if present), allowing p53 to enter the nuclei of the cells and trigger downstream apoptosis.
**Hypothesis** – p53 is cytoplasmically sequestered by mortalin in IMR-32 cells, and treatment with MKT-077 will free p53 from this sequestration, causing it to enter the nucleus and trigger apoptosis.
**Materials and Methods**

**Cell Culture** – A frozen vial of IMR-32 cells was obtained from the American Type Culture Collection (ATCC), and they were immediately reanimated and cultured in T-25 flasks. After the first few passages, sufficient cells were obtained to conduct MKT-077 treatments and the rest were cryopreserved for future use. Eagle’s Minimum Essential Medium (EMEM) was used with 10% fetal bovine serum (FBS) and 0.2% gentamicin as supplements. The conditioned medium was changed once every two to three days, and the cells were passaged once per week. The cells were cultured in an incubator at 37 °C, 5% CO₂, and 80% humidity. Their growth was monitored with an inverted microscope.

For passaging, the conditioned medium was aspirated, and the cells were washed with Hank’s Balanced Salt Solution (HBSS) for one minute before incubation with trypsin-EDTA for one to two minutes. Then, a double volume of fresh medium was used to neutralize the trypsin, and the cell suspension was centrifuged for five minutes at 1200 rpm. The supernatant was aspirated, and the cell pellet was resuspended in fresh medium. Cell number was determined using an automated cell counter prior to seeding into T25 flasks.

All cell culture work, including the MKT-077 treatments described below, was performed in a sterile biosafety cabinet.
**MKT-077 Treatments** – Cells were treated at five final concentrations of MKT-077. 5 mg of the MKT-077 was received in powdered form and all of it was initially dissolved in 1mL of sterile dimethyl sulfoxide (DMSO) to yield a 11.574 mM stock solution of MKT-077 in DMSO. Six T25 flasks were then seeded during a passage with $2.5 \times 10^6$ cells each, and cultured identically until 35% confluency was reached. Immediately prior to each treatment, the MKT-077 stock solution was then added to tubes containing fresh medium, producing the final concentrations (2 µM, 4 µM, 6 µM, 8 µM, and 10 µM) for treatments. DMSO was added to each tube in order to equalize the concentration of DMSO across all tubes, including the control tube (0 µM MKT-077). The final concentration of DMSO was under 0.1% (considered to be physiologically inactive). The tubes were then vortexed before the medium containing MKT-077 was added to each T25 flask containing cells.

The cells were incubated with MKT-077 at culture conditions for 24 hours. After the incubation period, the flasks were observed under the inverted microscope. The method used for cell dissociation was similar to the passaging protocol described above in the “Cell Culture” section, but with two important modifications. First, due to the significant number of floating cells, the conditioned medium and the HBSS used for the wash were saved and pooled with trypsin-EDTA cell suspension. Second, following centrifugation and aspiration of the supernatant, the cell pellet was resuspended in phosphate buffered saline (PBS) rather than fresh culture medium.
An aliquot from each of the six cell suspensions was then tested using a viability assay (described below). Slides of the cells were also prepared for immunocytochemistry (described below) using poly-L lysine to coat the slides and a cytospin centrifuge to adhere the cells onto the slides (cells were centrifuged onto the slides at 2000 rpm for one minute).

**Trypan Blue Viability Assays** – 20 µL of cells from each sample was mixed with an equivalent volume of trypan blue dye. The mixture was then resuspended to determine the number of living cells (which excluded the trypan blue dye) and the number of dead cells (which could not exclude the trypan blue dye), using an automated cell counter.

**Immunocytochemistry for p53** – **Summary**: Following a series of blocking steps, p53 was labeled using an indirect immunocytochemical method (to increase sensitivity, since p53 is constitutively present in low concentrations in the cell) involving primary antibody, biotinylated secondary antibody, HRP-conjugated avidin (horseradish peroxidase), and a chromogenic HRP substrate. Cell nuclei were then counterstained using hematoxylin, and the slides were mounted and sealed, prior to visualization under a bright-field microscope.

**Methodological Note**: All washes performed during the protocol involved placing the slides in Coplin jars containing the wash solvent, and placing the Coplin jars on a rotator for five minutes. The wash solvent, unless specified otherwise, was Vectastain buffer.
(VSB; see Appendix A for composition). All incubations performed during the protocol were performed at room temperature, and involved placing the slides in elevated positions “hydration chambers”: closed petri dishes containing moistened filter paper.

**Permeabilization and Blocking:** Cells were initially fixed and permeabilized by immersing the prepared slides in Coplin jars containing pre-cooled (4 ºC) acetone for ten minutes, and then washed with VSB. To block interference by endogenous biomolecules, the cells were then incubated with a peroxidase blocking solution for ten minutes, and then washed with VSB. Afterwards, an avidin/biotin blocking kit was used that contained ready-to-use solutions of avidin and biotin: the slides were incubated with avidin for fifteen minutes, and then washed with VSB, before they were incubated with biotin for fifteen minutes, and then washed with VSB.

**Indirectly Labeling p53:** Following the blocking steps, the slides were incubated with the primary antibody (DO-1, a mouse anti-human monoclonal IgG$_{2a}$ antibody for p53) diluted 1:50 in VSB for thirty minutes, and then washed with VSB. This was followed by an incubation with biotinylated secondary antibody (horse anti-mouse IgG) diluted 1:200 in VSB for thirty minutes, followed by another wash with VSB. The slides were then incubated with HRP-conjugated avidin (horseradish peroxidase) diluted 1:1000 in PBS for thirty minutes, and then washed twice with VSB. Finally, for visualization, DAB substrate (3,3'-diaminobenzidine) was used as the chromogen, producing a dark brown
color upon reaction with HRP. The cells were incubated with this substrate for eight minutes, and then washed in double deionized water.

**Counterstaining and Mounting:** To counterstain the cell nuclei blue, hematoxylin was applied to the slides for five to ten seconds, and they were then dipped in double deionized water for ten seconds. The slides were then mounted using a permanent mounting medium, cover-slipped, and sealed. The slides were stored in the dark until they were visualized and imaged under a bright-field microscope.

**Negative Controls:** For each sample, a negative control was also included to check for non-specific staining. The steps used for the negative control slides were identical to those described above, except that plain VSB was used in the place of primary antibody. Following that step, negative control slides and experimental slides were kept in separate Coplin jars and hydration chambers.
Results

Observations of Cells – Following the 24-hour treatment period: In flasks containing cells treated with MKT-077, the medium was turbid (more so at higher concentrations of MKT-077), and observations under the microscope revealed that many of the cells were floating (more at higher concentrations of MKT-077). For the untreated flasks, by comparison, the medium was clear, and most of the cells were firmly adherent in a monolayer at the base of the flask.

Trypan Blue Viability Assay Results – Based on the viability assay results returned by the automated cell counter, the percentage of unadjusted live cells was calculated by dividing the number of live cells by the number of total cells. The percentage of adjusted live cells was then calculated by setting the untreated control at 100% live cells for each replicate and expressing relative to it the results for the rest of that replicate's concentrations.
Figure 1: Unadjusted viability assay results, showing percent of live IMR-32 cells over concentration of MKT-077 in µM. Three replicates of the experiment were performed, and the data shown represents the mean ± SD for each concentration.
Figure 2: Adjusted viability assay results, showing percent of live IMR-32 cells over concentration of MKT-077 in μM. Three replicates of the experiment were performed, and the data shown represents the mean ± SD for each concentration.
Immunocytochemistry Results – Immunocytochemistry was performed on a total of twelve slides: an experimental slide and a negative control slide for each of five treatment concentrations, along with the untreated control. Each slide was visioned using the bright-field function of a Zeiss Axioplan 2 microscope, and four micrographs (all shown below) were taken for each slide: two at 400x magnification, and two at 1000x magnification.
Figure 3: Untreated (0 µM MKT-077) IMR-32 cells stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. EXPERIMENTAL SLIDE.
Figure 4: Untreated (0 µM MKT-077) IMR-32 cells stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. NEGATIVE CONTROL SLIDE.
Figure 5: IMR-32 cells treated with 2 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification.

EXPERIMENTAL SLIDE.
Figure 6: IMR-32 cells treated with 2 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. NEGATIVE CONTROL SLIDE.
Figure 7: IMR-32 cells treated with 4 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification.

EXPERIMENTAL SLIDE.
Figure 8: IMR-32 cells treated with 4 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. NEGATIVE CONTROL SLIDE.
Figure 9: IMR-32 cells treated with 6 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification.

EXPERIMENTAL SLIDE.
Figure 10: IMR-32 cells treated with 6 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. NEGATIVE CONTROL SLIDE.
Figure 11: IMR-32 cells treated with 8 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. EXPERIMENTAL SLIDE.
Figure 12: IMR-32 cells treated with 8 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. NEGATIVE CONTROL SLIDE.
Figure 13: IMR-32 cells treated with 10 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification.

EXPERIMENTAL SLIDE.
Figure 14: IMR-32 cells treated with 10 µM MKT-077 stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs from two fields on the slide (top and bottom rows) were taken at both 200x (left) and 1000x (right) magnification. NEGATIVE CONTROL SLIDE.
Figure 15: IMR-32 cells treated with various concentrations of MKT-077 (2 µM – 10 µM), and untreated (control) cells, stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs were taken at 1000x magnification. EXPERIMENTAL SLIDES.
Figure 16: IMR-32 cells treated with various concentrations of MKT-077 (2 μM – 10 μM), and untreated (control) cells, stained for p53 (dark brown). The nucleus is counterstained blue. Micrographs were taken at 1000x magnification. NEGATIVE CONTROL SLIDES.
Discussion

Conclusion – The hypothesis for the current study is supported by the data presented in the Results section. According to the immunocytochemistry micrographs for the untreated group (Figure 3), p53 appeared to be cytoplasmically sequestered in IMR-32 cells. Treatment with MKT-077 then resulted in p53 translocation to the cells’ nuclei, and—at higher concentrations of MKT-077—increased concentrations of p53 were observed within nuclei (Figure 15). Negligible amounts of staining are visible in the negative control slides compared to the experimental slides (Figure 16), indicating that the staining visible in the experimental slides is indeed specific for p53 and is not substantially labeling something else in the cells.

In addition, the viability assays (Figure 2) showed that MKT-077 caused death in IMR-32 cells, and that higher concentrations of MKT-077 caused significantly greater numbers of cells to die. Because p53 can be seen moving from the cytoplasm into the nucleus as MKT-077 is introduced, and MKT-077 is a known inhibitor of mortalin (Wadhwa et al., 2000), disruption of the p53-mortalin complex is a likely mechanism of MKT-077’s cytotoxic activity towards IMR-32 cells. It is therefore likely that the p53-mortalin complex is present in IMR-32 cells and responsible for the cytoplasmic sequestration shown by the micrographs (Figure 3), and that MKT-077 is freeing p53, allowing it to enter the nucleus and induce apoptosis in the cells transcriptionally. Future experiments that would confirm this mechanism are proposed below in the “Next Steps” section.
There is, however, one important thing to note about the trypan blue viability assays as they relate to the mechanism of cell death. Intact cell membranes exclude trypan blue, and so the dye only infiltrates (and marks as dead) cells that have compromised membrane integrity. The integrity of the cell membrane is compromised in necrosis, but not in apoptosis. However, apoptotic bodies—which would normally be phagocytized by a macrophage in vivo as the coup de grâce of apoptosis—eventually progress to necrosis and lose membrane integrity in vitro, in the absence of macrophages. This process is known as secondary necrosis (Silva and Nebreda, 2010). Therefore—while the viability assays indicate that cell death is occurring following treatment with MKT-077, and that it is occurring at greater levels when greater concentrations of MKT-077 are used—they do not distinguish between apoptosis and necrosis (Martin and Henry, 2013).

Additionally, given that MKT-077 is known to exhibit other means of toxicity—in particular, mitochondrial damage leading to necrosis—the mechanism proposed above involving the p53-mortalin complex is not the only possibility (Modica-Napolitano et al., 1996). However, when the viability assay data is combined with the data from immunocytochemistry (which shows that MKT-077 caused p53 to enter the nucleus of IMR-32 cells), the proposed mechanism involving the p53-mortalin complex does appear to be a likely possibility.
**Next Steps** – Additional experiments can aid in determining whether the true mechanism of MKT-077’s cytotoxic activity towards IMR-32 cells involves disruption of the p53-mortalin complex. Apoptosis assays, such as TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays or Annexin V assays, can be used to demonstrate that cell death is occurring via apoptosis, thereby ruling out necrotic mechanisms as possibilities. If performed, the results of these assays can be measured qualitatively via microscopy, or quantitatively via flow cytometry. Additionally, immunocytochemistry can be further used to dually-stain mortalin and p53, microscopically showing that they are co-localized in the same cellular compartment within IMR-32 cells before MKT-077 treatment, and in separate cellular compartments after MKT-077 treatment, when p53 dissociates and migrates into the nucleus.

Another approach would be to use a nuclear and cytoplasmic extraction kit, which can isolate nuclear and cytoplasmic proteins into separate fractions. Co-immunoprecipitation—in which p53 would be precipitated out of solution, and immunoblotting would be used to check for the presence of both p53 and mortalin—can then be used on all cytoplasmic fractions to confirm that p53 and mortalin are originally bound together, and that treatment with MKT-077 causes them to dissociate. Immunoblotting can additionally be used on all nuclear fractions, which would theoretically show that nuclear concentration of p53 increases with increasing concentrations of MKT-077.
Moreover, testing other known mortalin inhibitors—such as withanone, a chemical extracted from *Ashwagandha*, a plant used in traditional Indian Ayurvedic medicine—on the IMR-32 cells would provide strong supporting evidence that disrupting the p53-mortalin complex is a good way to selectively target cancer cells that are characterized by it (Grover et al., 2012).

**Future Work** – Future work focusing on the p53-mortalin complex in neuroblastoma can utilize other human neuroblastoma cell lines, or samples of neuroblastoma derived directly from patients. The latter is difficult to obtain and study, due to the lack of availability and uniformity, but data based on patient samples would provide evidence about the p53-mortalin complex in this cancer that is more strongly and directly applicable to medicine, and to the goal of finding therapeutic strategies that would combat neuroblastoma. In a broader context, future work focusing on the p53-mortalin complex can employ the experimental design and methodologies similar to those used here, and those described in the “Next Steps” section above, in order to study the complex in other cancers.

**Applications** – Based on the results and conclusions of the current study, mortalin inhibitors, if investigated further, could potentially be used as a selectively cytotoxic component of a therapeutic strategy in the treatment of neuroblastomas where the p53-mortalin complex is present. In general, the treatment of cancer is made especially difficult by the challenge of distinguishing cancer cells from healthy cells. Many cancer
therapies that are currently in use target all actively dividing cells, and tend to have severe negative side effects as a result. The p53-mortalin complex, however, is a major factor that contributes to the development of cancers that are characterized by it; a therapeutic agent that targets it could therefore theoretically be highly selective for cancer cells, and would thus be likely to have fewer negative side effects than chemotherapies that are currently in use.

MKT-077 has been tested previously in a Phase I clinical trial, and the study was halted after MKT-077 caused damage to patients' kidneys (Propper et al., 1999). It probably won’t be used clinically as a therapeutic agent, unless a lower dose could somehow be made effective. However, the results of the current study showed that the p53-mortalin complex is a viable target, and that other mortalin inhibitors—such as, for example, withanone, mentioned above in the “Next Steps” section—could potentially be viable therapeutic agents to selectively target cells of cancers characterized by this complex.
References


Appendix A: Materials Reference

Suppliers

- ATCC (American Type Culture Collection)
- Baker (The Baker Company)
- BioRad (Bio-Rad Laboratories)
- Corning (Corning Incorporated)
- EMS (Electron Microscopy Sciences)
- Eppendorf (Eppendorf North America)
- GE (GE Healthcare Life Sciences)
- Gilson (Gilson Incorporated)
- Markwins (Markwins Beauty Products)
- Olympus (Olympus Corporation)
- SCBT (Santa Cruz Biotechnology)
- Sigma (Sigma-Aldrich)
- Thermo (Thermo Fisher Scientific)
- USAS (USA Scientific Incorporated)
- Vector (Vector Laboratories)
- VWR (VWR International)
- Zeiss (Carl Zeiss)
Materials

CELL CULTURE
- IMR-32 Cells: ATCC #CCL-127 (LOT: #59587034)
- Vented Cell Culture Flasks (T-25, T-75): Corning #CLS430639, Corning #430641
- EMEM: Sigma #56416C-10L
- Fetal Bovine Serum (FBS): Sigma #F2442
- Gentamicin: Thermo #15710-064
- Sterile Filters: Corning #430767
- Hank’s Balanced Salt Solution (HBSS): Thermo #14025-076
- Trypsin-EDTA: GE #SV30031.01
- Cryogenic Vials: Thermo #12-567-501
- Freezing Containers: Thermo #5100-0001
- CO₂ Incubator (Napco 8000WJ): Thermo #3423
- Biosafety Cabinet: Baker #SG603A-HE
- Inverted Microscope: Olympus IMT-2 #04590
- General Centrifuge: Eppendorf Centrifuge 5702
- Water Bath: Thermo Isotemp 2320
- Centrifuge Tubes (15 mL): Thermo #362694
- Automatic Pipetter: Eppendorf Easypet 3
- Serological Pipets (1 mL, 5 mL): Thermo #13-676-10G, Thermo #13-676-11D
- Pasteur Pipets: Thermo #13-678-6B

MKT-077 TREATMENTS
- MKT-077: Sigma #M5449-5MG
- Dimethyl Sulfoxide (DMSO)
- Vortexer: Thermo #14-955-151
- Pipetters (Eppendorf Research Plus P10, P200, P1000): Eppendorf #3124000016, Eppendorf #3124000083, Eppendorf #3124000121
- Pipet Tips (P10, P200, P1000): Gilson #F161631, Thermo #02-707-500, Thermo #02-707-507

TRYPAN BLUE VIABILITY ASSAYS
- Trypan Blue Stain: Thermo #15250-061
- Automated Cell Counter: BioRad TC-20

IMMUNOCYTOCHEMISTRY FOR P53
- Vectastain Buffer: In 1 L of deionised water, mix 9 mL PBS + 2.76 g sodium phosphate heptahydrate dibasic + 0.26 g sodium phosphate monobasic
- Phosphate Buffered Saline (PBS): Thermo #10010-023
- Sodium Phosphate Heptahydrate Dibasic: SCBT #sc-203402A
- Sodium Phosphate Monobasic: Thermo #S369-500
- Acetone: VWR #97065-060
- Peroxidase Blocker (BLOXALL): Vector #SP-6000
- Avidin/Biotin Blocking Kit: Vector #SP-2001
- Primary Antibody (DO-1, Mouse Anti-Human p53): SCBT #sc-126
- Secondary Antibody (Horse Anti-Mouse IgG): Vector #BA-2000
- HRP-Conjugated Avidin (Horseradish Peroxidase): Vector #A-2004
- DAB Substrate (3,3’-diaminobenzidine): Vector #SK-4105
- Hematoxylin: Vector #H-3404
- Permanent Mounting Medium (ProLong Gold Antifade): Thermo #P36934
- Sealant (Wet n’ Wild Nail Protectant): Markwins #450B
- Microscope: Zeiss Axioplan 2
- Cytospin Centrifuge: Thermo Shandon CytoSpin 4
- Multi-Purpose Rotator: Thermo #2314-1CEQ
- Glass Slides: Thermo #5991065
- Cover Slips: VWR #48380-080
- Slide Storage Book: EMS #71520
- Poly-L-Lysine: Sigma #P4707
- Filter Paper: GE #1003-125

Microtubes (0.5 mL, 1.5 mL): USAS #1405-2600, USAS #1415-2600