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Activating the MSH2/MSH6 Apoptotic Pathway in Cancer Cells Using Non-Reserpine

Compounds

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in

Biology

By

Jacob Mauceri

Under the mentorship of Andrew Diamanduros

ABSTRACT

DNA mismatch repair (MMR) is a system that is highly conserved in both prokaryotes and eukaryotes. The heterodimer MutS α and a suite of associated proteins are essential in the recognition and repair of DNA afflicted with mispaired bases and short insertion/deletion loops, but is also implicated in funneling damaged cells towards apoptosis via a key conformational change that can be bound specifically by the small molecule reserpine. Molecular dynamics modeling and virtual screening were used to identify additional small molecule novel ligands with the predicted ability to selectively bind this “death” conformation of MutS α . These novel ligands were demonstrated to possess cytotoxicity similar to that of reserpine. As MMR deficiency has been demonstrated to confer a degree of resistance to some chemotherapeutic agents, exploiting this novel apoptotic pathway may prove to be a valid niche treatment in particular classes of cancers in which MMR proteins have been mutated.

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Introduction and Literature Survey

In both prokaryotes and eukaryotes, DNA replication is a tightly controlled and highly regulated process. This essential stage in the lifecycle of a cell is regulated by a suite of proteins that, collectively, unwind the DNA to be replicated, replicate the parent DNA, and terminate the replicative process once the daughter strands have been successfully polymerized (Frouin, Montecucco, Spadari, & Maga, 2003). Maintaining the integrity of the DNA molecule's primary structure during replication is an essential process that is accomplished by a variety of biochemical pathways, such as the mismatch repair pathway.

The DNA mismatch repair (MMR) system is responsible for the detection and resolution of two major forms of polymerase errors: partially extrahelical heterogenous insertion/deletion loops (IDLs), and mismatched nucleotide bases (Jiricny, 2006). Deficiencies in MMR proteins most evidently present as increased microsatellite instability, a hypermutable phenotype that increases susceptibility to various forms of cancers, but particularly colorectal cancers (Boland and Goel, 2009). Additionally, deficient MMR systems may play a greater role in the decreased cytotoxicity of specific chemotherapeutic agents, such as the broad spectrum chemotherapeutic drug cisplatin, where deficiencies in the MMR pathways of cancer cells increased their resiliency to the drug 2-4 fold (Irving and Hall, 2001).

The protein MutS (mutator S) and its eukaryotic homologs (mutator S homolog 2, MSH3, and MSH6) are responsible for the initial recognition of DNA mismatches and the consequent formation of the primary protein/DNA heterodimer complex, which

then recruits other proteins and cofactors essential to the mismatch repair process (Jiricny, 2006). Specifically, MutS dimerizes to form a homodimer. In the event of irreconcilable DNA damage, these proteins (MutS and its eukaryotic homologs) possess additional regulatory functionality by promoting the activation of the caspase-mediated apoptotic pathway (Vasilyeva, 2009). Just how the prokaryotic MutS and the eukaryotic

Table 1. A list of human MutS homologs, their component subunits, and their known function. Obtained from Jiricny (2006).

Complex	Components	Function
MutS α	MSH2 + MSH6	Recognition of base-base mismatches and short IDLs
MutS β	MSH2 + MSH3	Recognition of longer IDLs
MutL α	MLH1 + PMS2	Formation of ternary complex with DNA-bound MutS α
MutL β	MLH1+ PMS1	Unknown

MutS α/β heterodimeric complexes participate in the apoptotic caspase-signaling cascade remains subject to debate, with two competing hypothesis dominating academic contention. The “futile repair cycle” hypothesis posits an indirect role of MMR proteins in activating caspase-mediated apoptosis – instead of directly activating proteins within the caspase cascade, apoptosis is triggered as a result of DNA strand breakage formation following repeated “futile” repair attempts by the MMR system in

which mismatch damage persists. It is then through these standing strand breaks that the apoptotic pathway is initiated (Roos and Kaina, 2006). Conversely, the “direct signaling” hypothesis propounds a dual functionality for at least the MutS α complex in eukaryotes. According to this hypothesis, cell death is initiated by the MMR proteins themselves, particularly MutS α , through direct signaling that results in the activation of the caspase-mediated apoptotic-signaling cascade. (Roos and Kaina, 2006). This then suggests that there exist two distinct conformations for the MutS α heterodimer – a pro-repair conformation in which DNA repair is promoted, and an alternative “death” conformation in which the protein abandons its repair function and instead promotes cell death via an apoptotic-signaling cascade (Salsbury, Clodfelter, Gentry, Hollis, and Scarpinato, 2006). Vasilyeva et al. (2009) suggested that both mechanisms could, and likely do, occur. It is further suggested that which conformation – and consequently, which functionality – MutS α assumes is dependent on the substrate located within the protein’s DNA binding groove; mismatched DNA promotes a “repair” conformation and response, and damaged DNA promotes a “death” conformation and response (Ling, 2004; Salsbury et al., 2006). As each pathway acts independently of the other, and is ostensibly substrate dependent, it is possible to selectively activate the apoptotic pathway with novel ligands, which has been demonstrated with the drug reserpine, and its derivatives.

Reserpine is an FDA approved indole alkaloid drug isolated from the Indian snakeroot (*Rauwolfia serpentine*), and used historically as an antihypertensive in the regulation of blood pressure. Vasilyeva et al. (2009) identified reserpine as a possible

novel ligand capable of selectively binding the MutS α complex to a proposed “death conformation” via molecular dynamics simulation and virtual screening. The x-ray structure of the MutS complex from *Escherichia coli* complexed with DNA (of which the MutS α is a homolog) was used as a model for 3D virtual analysis of the active site in conjunction with novel ligands. The molecular dynamic simulation was used to estimate the inhibition constant K_i of novel ligands. Using this method, reserpine was identified as a potential novel ligand with a hypothetical twenty-fold affinity for the proposed apoptotic conformation of the MutS α heterodimeric complex over the repair conformation. Reserpine has been demonstrated to induce apoptosis via the MSH2/MSH6-mediated apoptotic pathway *in vitro*; however, the necessary concentrations needed for anti-tumour activity causes dangerous hypotension *in vivo*, and it is therefore not a viable chemotherapeutic agent in humans. Reserpine’s ability to effectively induce MMR-dependent apoptosis in cancer cells is, however, a proof-of-concept that virtual analysis of molecular dynamics is an effective approach in identifying possible novel ligands to bind proteins to specific desired conformations (Vasilyeva et al., 2009). Thus, it should be possible to generate additional small-molecule novel ligands capable of binding the MSH2 subunit to its proposed “death” conformation.

Protein/DNA Interaction

Just as prokaryotic DNA replication varies from its eukaryotic counterpart, so too does the MMR machinery vary in both models. In mammalian cells, different MMR machinery participates in the repair/abort pathways dependent on the type of DNA

damage encountered (Acharya, Wilson, Gradia, Kane, Guerrette, Marsischky, Kolodner, and Fishel, 1996). The heterodimeric MutS α initiates the repair of single-base mismatches and short insertion/deletion loops (IDLs) of one or two extrahelical nucleotides. Insertion/deletion loops that contain two or more extrahelical nucleotides are recognized by the related complex MutS β , which is a heterodimer of MSH2 and MSH3 (Acharya et al., 1996; Palombo, Iaccarino, Nakajima, Ikejima, Shimada, and Jiricny, 1996). In this way, the mammalian MMR system exhibits partial redundancy. The initial binding of the MutS α complex to heterogenetic duplex DNA is mediated by the two DNA binding domains (“clamp domains”) of the MutS α complex. Each subunit possesses a clamp domain, and it is only the clamp domain of the MSH6 subunit that actually contacts the nitrogenous bases of the DNA (and are thus the only part of the mechanism that is sequence-dependent) (Obmolova et al., 2000; Lamers et al., 2000).

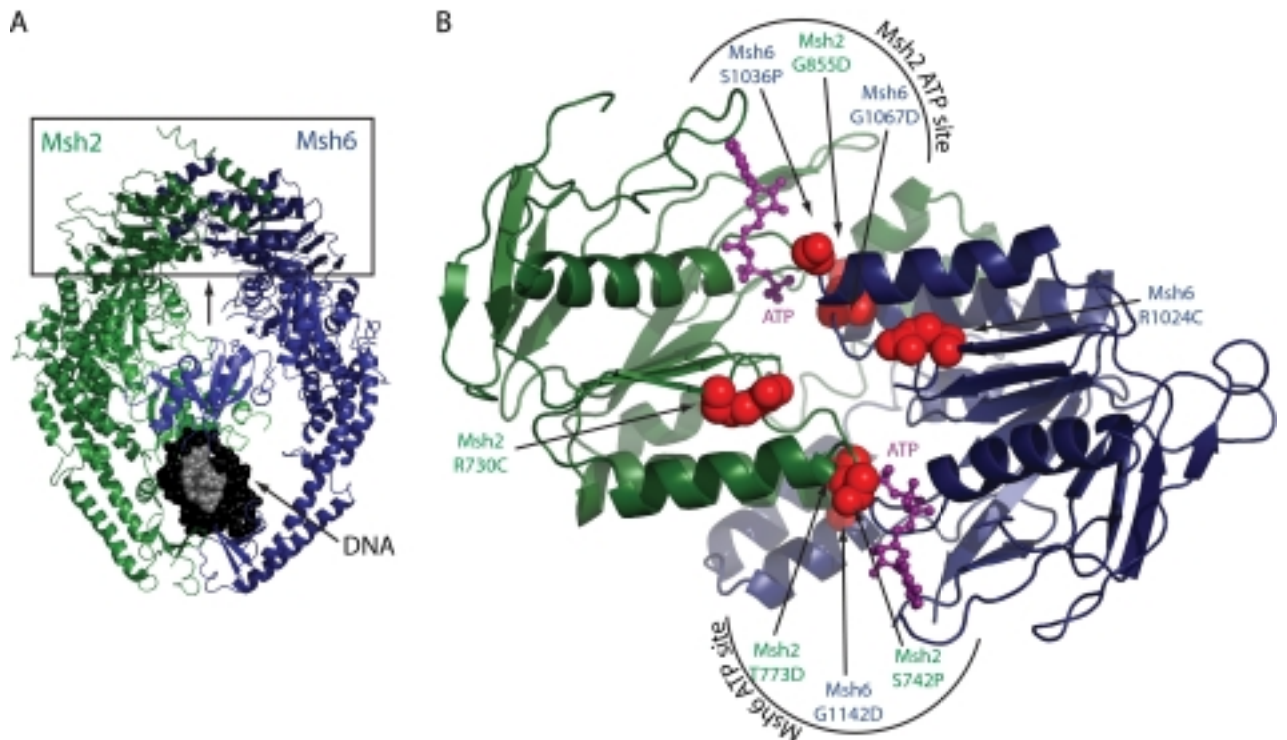


Figure 1. A. Ribbon model of the MSH2/MSH6 heterodimer in complex with heterogenous DNA (shown in black). B. View of the the complex's ATP binding domains, with ATP positioning included. Obtained from Hargreaves (2010).

This anchoring is achieved by the presence of a highly conserved Phe-X-Glu motif (where 'X' is typically a negatively charged amino acid) present exclusively in domain I of the MSH6 subunit of the MutS α complex, which approaches the daughter strand DNA from the minor groove at the nucleotide base 3' to the mismatch (Lamers et al., 2000). This protein/DNA interaction is ordinarily impossible due to electrostatic repulsion between the negatively charged side chains (the conserved glutamine and the variable amino acid 'X') and the negatively charged phosphate backbone of the duplex DNA, but a mismatch event widens the minor groove of the heterogenous duplex DNA, allowing these normally-repulsed side chains to occupy the groove with little difficulty, and results in a bending of the DNA by approximately 60° (Lamers et al., 2000; Jiricny, 2006).

This “kinked” DNA conformation, normally energetically unfavorable, is stabilized by its interaction with the rest of the domains of the protein, which form a network of hydrogen bonding and salt bridges. Additionally, the bending of the DNA 60° towards the major groove causes a transient puckering of the nucleotide sugars from the C2'-endoconformation that is typical of B type DNA to the more energetically favorable C3'-endoconformation that characterizes A type DNA in the nucleotide bases immediately surrounding the site of the base-base mispair (Nag, 2007; Obmolova et al., 2000). However, these interactions occur only between the protein and the phosphate backbone – the binding and stabilizing of the DNA around the mispair is sequence-independent (Lamers et al., 2000; Obmolova et al., 2000; Nag, 2007).

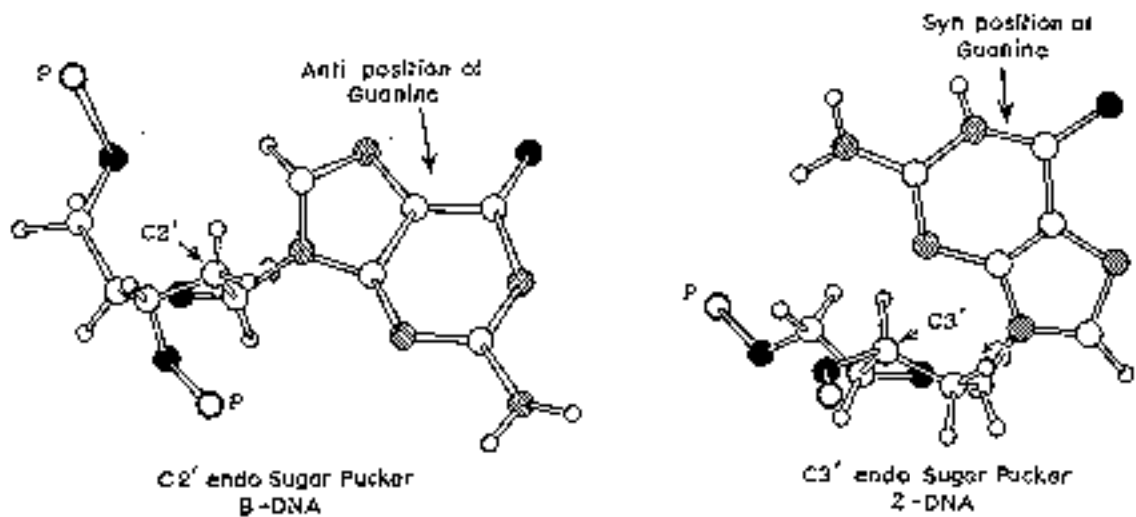


Figure 2. Structural alteration between the C2' and C3' endoconformations of the deoxyribose sugar ring. Also included is the syn/anti positioning of the attached guanine base.

An Overview of the Repair Pathway

Once bound to the DNA at the site of the mismatch, the MutS α complex undergoes a conformational change heralded by the exchange of the ADP molecule present in the MSH6 subunit for an ATP molecule. The new conformation causes the intercalated Phe-X-Glu motif to release the DNA, allowing it to move along the DNA as a sliding clamp (Jiricny, 2006). This change in conformation from statically bound protein anchor to hydrolysis-independent sliding clamp is crucial for initiating the repair functionality of the pathway. Once this conformational transition has been completed, MutS α is free to slide along the DNA contour in either direction, although the direction in which it travels affects the downstream proteins that will be recruited and the subsequent directionality of strand degradation (Jiricny, 2006). The next step in the pathway involves the association of the MutS α complex with another MMR heterodimer, MutL α . This protein complex exists as a heterodimer of the MMR proteins MLH1 (MutL homolog-1) and PMS2 (post-meiotic segregation protein-1), and has been shown to complex with MutS α . The exact role of the resultant, likely transient ternary complex remains hitherto unclear (Plotz, Raedle, Brieger, Trojan, and Zeuzem, 2002; Plotz, Piiper, Wormek, Zeuzem, and Raedle, 2006; Jiricny, 2006). It is postulated that this interaction between heterodimers is necessary to mediate the ATP-dependent turnover of the MutS α complex, or/and alternatively mediate interactions between the functional MutS α subunits and associated MMR proteins at the strand excision site downstream of the mismatch (Plotz et al., 2002). Regardless, MutL α has been experimentally shown to be an integral component of the MMR repair pathway, as mice with the component

monomers knocked out ($PMS2^{-/-}$ and $MLH1^{-/-}$) phenotypically express significantly increased microsatellite instability and tumorigenesis (Marra and Jiricny, 2003). The next step of the pathway is dependent on both the direction in which the sliding clamp travels along the DNA after it has been complexed with $MutL\alpha$, and the presence of previously-formed strand breaks within the nascent daughter strand. As MMR functionality requires preexisting strand breaks *in vitro*, it is hypothesized that Okazaki fragment termini serve as these strand discontinuities *in vivo* (Ghodgaonkar, Lazzaro, Olivera-Pimentel, Artola-Borán, Cejka, Reijns, Jackson, Plevani, Muiz-Falconi, Jiricny, 2013). $MutS\alpha$ / $MutL\alpha$ clamps that move upstream along the DNA contour encounter the clamp-loading protein replication factor C (RFC) attached to the 5' terminus of the single-strand break. The sliding clamp then promotes the displacement of RFC from the DNA, and recruits the exonuclease EXO1 (exonuclease-1). EXO1 initiates the subsequent degradation of the daughter strand in the 5'→3' direction, with the resultant single-stranded DNA stabilized by replication protein A (RPA) (Jiricny, 2006). Once the mismatch has been successfully excised by EXO1, EXO1's exonuclease activity is simultaneously no longer promoted by $MutS\alpha$ and actively inhibited by $MutL\alpha$. Concurrently, DNA polymerase δ is promoted to load at the 3' terminus of the original strand break by its processivity factor proliferating cell nuclear antigen (PCNA). The newly-excised strand allows DNA polymerase δ to reattempt faithful DNA replication across the site of the previous mispair, and the remaining nick is ligated by DNA ligase I (Yang, 2000; Jiricny, 2006). This process is largely the same for $MutS\alpha$ / $MutL\alpha$ clamps that move downstream from the initial mispair, with reversed polarity. Upon diffusing

downstream, the MutS α / MutL α clamp first encounters a PCNA molecule bound to the 3' terminus of the Okazaki fragment, with an RFC molecule bound nearby. Upon contact with PCNA, the MutS α / MutL α clamp complex recruits EXO1 to excise nucleotides in a 3'→5' orientation – 5'→3' exonuclease activity is prevented by the downstream RFC molecule. Exonuclease activity continues upstream until the mispaired base has been removed, at which point EXO1 exonuclease activity is inhibited. RPA molecules stabilize the exposed single-strand while DNA polymerase δ loads at the site at which EXO1 exonuclease activity ceased (DNA polymerase δ possesses only 5'→3' replication activity). Finally, DNA ligase I seals the remaining nick (Yang, 2000; Jiricny, 2006).

MMR Role in Apoptosis Activation is Significant but Poorly Understood

Mismatch repair proteins have been repeatedly implicated in the activation of cell death (Lin, 2004; Jiricny, 2006; Vasilyeva et al., 2009), but how they interact with the apoptotic machinery of the cell remains poorly understood (Lin, 2004; Vasilyeva et al., 2009). Currently, two hypotheses are considered most likely: the futile repair cycle model and the direct signalling model, with evidence suggesting that both pathways may be used situationally by the cell (Salsbury et al., 2006). The futile repair cycle hypothesis suggests that repeated unsuccessful repair attempts lead to standing strand degradation, which initiates apoptotic pathways and funnels the cell towards death (Mello, Acharya, Fishel, and Essigmann, 1996). Conversely, the direct signalling hypothesis proposes a more involved role by the MMR system in initiating eukaryotic cell death in which a conformational change in the mismatch recognition complex MutS α results in the protein actively recruiting factors that funnel the cell towards

apoptosis (Salsbury et al., 2006). Importantly, Vasilyeva et al. (2009) established that this “death” conformation could be selectively activated by small-molecule ligands, and that resultant cell death does proceed through the caspase-mediated apoptotic pathway. Additionally, it has been shown that cells with repair-deficient MMR proteins are still susceptible to cisplatin-induced cytotoxicity, which further lends credence to two discrete functions of the MutS α complex (Lin et al., 2004; Salsbury et al., 2006).

Materials and Methods

Virtual screening and molecular dynamic simulations were performed in collaboration with Wake Forest University. AutoDock 3.0 was used to screen prospective compounds identified by molecular dynamics simulation analysis, and a list of potential molecules calculated to hypothetically bind the MutS α “death” conformation with a high degree of fidelity (according to the inhibition constant, K_i) was generated. Protocol for the dynamics simulation and virtual screening was as described elsewhere (Morris, Goodsell, Halliday, Huey, Hart, Belew, and Olson, 1998; Salsbury et al., 2006).

Prospective compounds were then subjected to the colorimetric CellTiter 96[®] AQueous One Solution Cell Proliferation Assay[®] (MTS assay) to determine cell viability. PC3 prostate cancer cells were cultured in standard growth media and transferred to 96-well plates. CellTiter 96[®] AQueous One Solution Reagent (containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES)) were added to the cells and incubated for 24 hours. The drugs to be tested in addition to a reserpine control were dissolved in an appropriate solvent (typically DMSO

and acetic acid), and a stock solution of drug with sufficient volume for the amount of plated cells for any given assay was prepared. Solvents used were tested for cytotoxicity. A serial dilution was then performed to generate 1/64, 1/32, 1/16, 1/8, and 1/4 dilutions from the stock, and the last aliquot left as a control, receiving only the DMSO/acetic acid solvent. The drug dilutions were then added to the plate in triplicates, incubated for 1-1.5 hours, and the absorbance read at 490nm by a 96-well plate reader. The amount of formazan product formed as the MTS is metabolized correlates proportionally to the amount of viable cells left in culture. This data was then used to generate a “kill curve,” or a curve illustrating the rate of cancer cell death for each concentration of tested drug, and compared to the reserpine control curve to determine comparative efficacy.

Results

Molecular Analysis Identified Reserpine-like Compounds

Following molecular analysis via virtual screening and molecular dynamics simulation, two commercially available drugs were identified as being hypothetically able to bind the MutS α “death” conformation in the same way as reserpine. These two drugs – C19H15N5O3S and P701100, shortened to “C19” and “P7” respectively – were used to perform a cell viability assay, where their induced cytotoxicity was compared to the reserpine control.

Small Molecule Novel Ligands Express Reserpine-like Cytotoxicity in vitro

Molecular modeling suggested that P7 and C19 would selectively bind the “death” conformation of the MutS α heterodimer much in the same way that reserpine does, and trigger cell death in a similar manner. Thus, we expected the cytotoxicity of these compounds to be at least as effective as reserpine. Indeed, experimental cytotoxicity of P7 and C19 proved to be just as effective as that of reserpine, with P7 performing slightly better at the highest concentration of drug (Figures 3, 4).

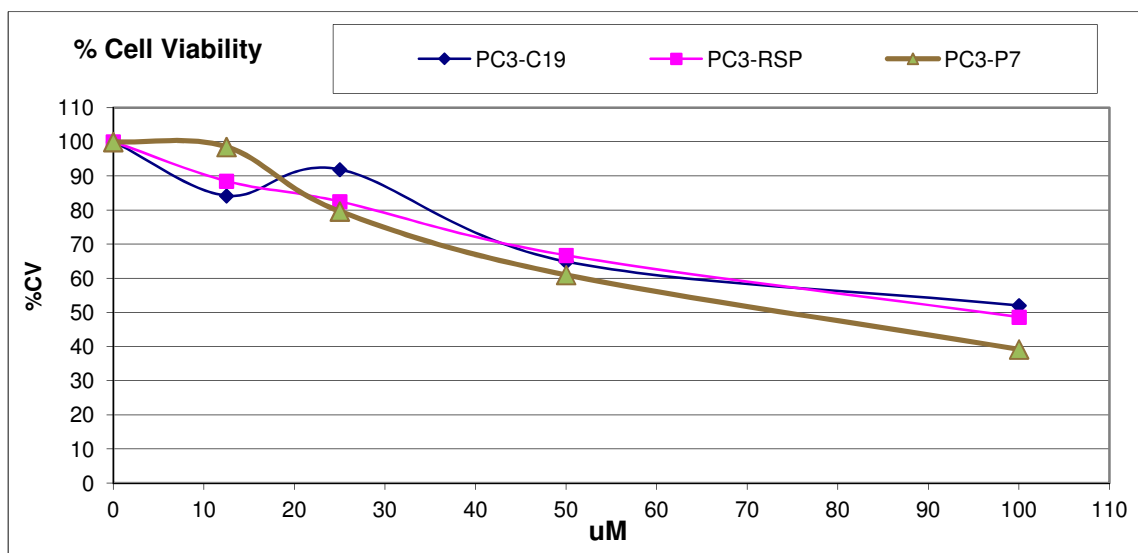


Figure 3. An MTS cell viability assay illustrates the cytotoxicity shared by the reserpine control and its behavioural analogs, P7 and C19, at increasing levels of drug concentration.

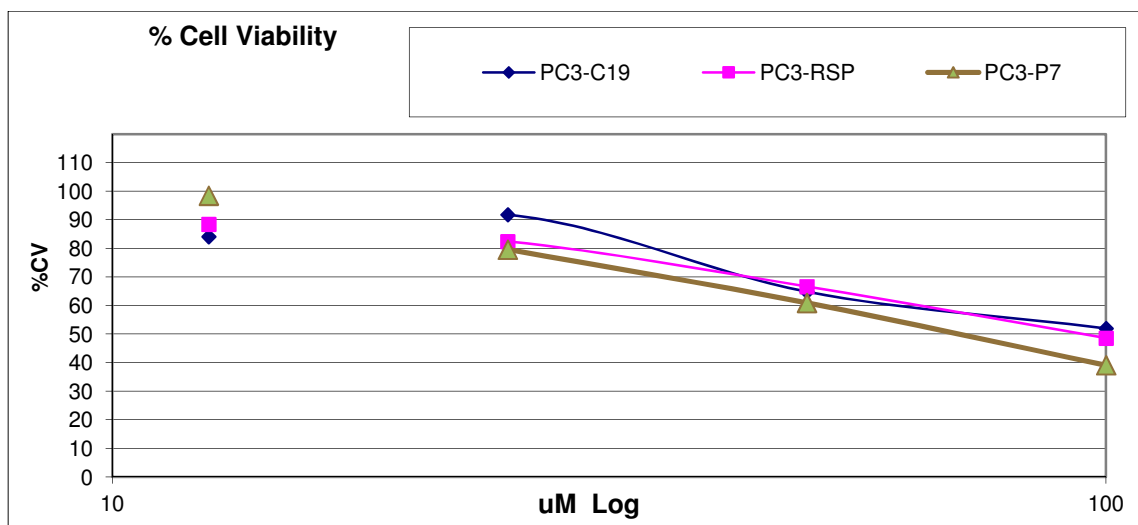


Figure 4. Log transformed version of Figure 3. Note: we observed small concentrations of drugs stimulating cell survival, resulting in a % cell viability greater than 100, which cannot be plotted.

Discussion

In this experiment, we confirmed molecular modeling and 3D virtual screening of proteins and known, indexed compounds to be an effective method in identifying novel ligands capable of selectively binding proteins to desired conformations. Indeed, we were able to identify a novel ligand (P7) that may prove to induce MutS α -mediated apoptosis more effectively than reserpine, and yet other compounds yet to have their 3-dimensional structure elucidated and indexed may prove to be more effective still. This would mean that a greater amount of cells could be killed with lower concentrations of drugs, which reduce the risk of serious side effects *in vivo* – the major limiting factor of reserpine and its derivatives as anything more than a niche chemotherapeutic.

More broadly, demonstrating the efficacy of a chemotherapeutic agent *in vitro* is the necessary precursor to identifying an agent that is viable *in vitro*; thus, by successful identification of novel and effective cytotoxic agents *in vitro*, we open the door to future *in vivo* testing of agents that may exploit a novel pathway not currently used clinically,

such as the MutS α -mediated apoptotic pathway. Indeed, since deficient MMR proteins have been implicated in conferring some degree of cytotoxic resistance to the cancer cells in which they are mutated by not triggering the apoptotic pathway via their naturally damaged DNA substrate (Irving and Hall, 2001), being able to bypass the DNA trigger with a novel ligand may be an effective niche treatment in some cancers. Of particular interest in that regard are colorectal cancers, in which MMR systems are commonly deficient (Boland and Goel, 2010). Exploiting the apoptotic functionality of MMR proteins may prove to be an effective niche treatment of this class of cancers.

Additionally, our results support the existence of the direct-signaling hypothesis. By identifying novel ligands that were predicted to fit a proposed pro-apoptotic conformation and subsequently observing cytotoxicity upon the treatment of cells with those ligands, we support the direct involvement of MMR proteins in funneling a cell with irreparable DNA damage towards apoptosis. However, additional caspase analysis needs to be performed to further substantiate MMR proteins initiating apoptosis.

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