UNIVERSITI PUTRA MALAYSIA

EFFECT OF COMBINATION THERAPY OF RUTHENIUM POLYPYRIDYL COMPLEX, $[Ru(dppz)_2(PIP)]^{2+}$ AND PARP INHIBITORS AGAINST SEVERAL CANCER CELL LINES

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By

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Faculty : Science

Ruthenium polypyridyl complexes (RPCs) have been clinically studied as promising anticancer agents in the last decades. The RPC, $[Ru(dppz)_2(PIP)]^{2+}$ or RuPIP where dppz = dipyrido[3,2-*a*:2',3'-*c*]phenazine and PIP = 2-(pheny)-imidazo[4,5-*f*][1,10]phenantroline has demonstrated anticancer properties where it was shown to stall DNA replication fork progression resulting in the initiation of DNA damage response (DDR) signaling which further lead to the inhibition of cell proliferation through G1/S-mediated cell cycle arrest. This has prompted us to study the rational combination of RuPIP alongside DDR inhibitors, particularly the inhibitors of poly (ADP-ribose) polymerase (PARP) to achieve synergistic effect in cancer cells. This is to enhance the therapeutic response to RuPIP in cancer cells while reducing the impact on normal cells. The cytotoxic effect of RuPIP and PARP inhibitors as single agents on several cancer cell lines (A549, MCF7, MDA-MB-231 and T24) were determined using MTT assay. RuPIP showed time-dependent reduction in the IC_{50} values meanwhile, both PARP inhibitors showed IC_{50} > 100 µM. All compounds showed IC_{50} > 100 µM on the normal NHDF cells. Drug combination study was carried out based on Chou and Talalay combination index (CI) method in which CI values were determined using Calcusyn and Compusyn software. Synergy (CI < 1) was observed with majority of RuPIP-olaparib combinations meanwhile, RuPIP-NU1025 resulted in a range of combination indices, ranging from synergism to antagonism. Importantly, the viability of normal cells observed for any combination tested was > 70%. Based on the average CI values, the synergistic combination (CI = 0.87) of 25 µM RuPIP alongside 25 µM NU1025 or 5 µM olaparib were chosen for further experiments. Cells ability to survive post-treatment and form colonies was investigated using clonogenic survival assay with single-agent treatments showed survival fractions (S.F) > 75%. Interestingly, combination treatments reduced cell survival with RuPIP-olaparib (S.F. < 2%) showed lower survival than RuPIP-NU1025 (S.F. < 57%). Besides, treatments with 25 µM RuPIP sensitize cells to olaparib where 60-fold reduction in IC_{50} value of olaparib was obtained for MCF7 (0.08 vs 4.75

μM) and 300-fold reduction was observed in MDA-MB-231 cells (0.06 vs 23.39 μM). Next, cell migration ability was investigated using cell scratch assay which revealed that RuPIP-olaparib combination significantly ($P < 0.001$) reduced cell migration with 40% reduction in wound closure was observed compared to control. The flow cytometric analysis on cell cycle distribution revealed that the combination treatment resulted in enhanced cell cycle arrest at G1/S phase in A549 cells (17.1% increase compared to control). Whereas both MCF7 and MDA-MB-231 cells were arrested at G2/M phase (19.7% and 20.4% increase compared to control, respectively). Subsequently, Annexin V-FITC assay showed that the combination treatment significantly ($P < 0.001$) increased the percentage of apoptotic cells with 25%, 30% and 31% increase compared to control in A549, MCF7 and MDA-MB-231 cells, respectively. These resultant cell deaths are associated with significant ($P < 0.001$) increase in the accumulation of double-strand breaks (DBSs) DNA damage with 45% increase in the percentage of cells with γH2AX foci compared to control. These findings established that RuPIP showed synergy with PARP inhibitors in several cancer cell lines with reduced impact on normal cells.

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KESAN KAEDAH RAWATAN GABUNGAN KOMPLEKS RUTENIUM POLIPIRIDIL, $[Ru(dppz)_2(PIP)]^{2+}$ DAN PERENCAT PARP TERHADAP BEBERAPA JENIS SEL KANSER

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Kompleks rutenium yang berasaskan ligan polipiridil (RPCs) mempunyai potensi sebagai ubat antikanser dan telah dikaji secara klinikal sejak sedekad yang lalu. Peranan RPC, $[Ru(dppz)_2(PIP)]^{2+}$ atau RuPIP di mana dppz = dipirido[3,2-*a*:2',3'-*c*]fenazin dan PIP = 2-fenilimidazo[4,5-*f*][1,10]fenantrolin) dalam terapi antikanser telah terbukti di mana ianya dapat melengahkan perkembangan cabang pereplikaan sewaktu replikasi DNA serta mengakibatkan pengaktifan tindak balas kerosakan DNA (DDR) yang kemudiannya merencatkan pertumbuhan sel melalui penangkapan kitaran sel pada fasa G1/S. Perkara ini telah mendorong kami untuk membuat kajian secara rasional dengan menggunakan gabungan RuPIP dan perencat DDR, terutamanya perencat polimerase poli (ADP-ribosa) (PARP) untuk menghasilkan kesan sinergistik bagi memperluas kesan terapeutik RuPIP serta mengurangkan kesan sitotoksik terhadap sel normal. Kesan sitotoksik yang dihasilkan oleh RuPIP dan perencat PARP sebagai agen tunggal terhadap beberapa jenis sel kanser (A549, MCF7, MDA-MB-231, dan T24) telah dikaji menggunakan ujian MTT. RuPIP menunjukkan penurunan nilai IC_{50} yang bergantung pada tempoh inkubasi, manakala kedua-dua perencat PARP menunjukkan IC_{50} > 100 µM. Semua sebatian menunjukkan IC_{50} > 100 µM terhadap sel normal NHDF. Seterusnya, kajian gabungan ubat-ubatan telah dinilai dengan menggunakan kaedah Chou dan Talalay kombinasi indeks (CI) dan pengiraan nilai CI telah ditentukan dengan menggunakan perisian Calcusyn dan Compusyn. Majoriti daripada gabungan RuPIP dan olaparib menghasilkan kesan sinergistik (CI < 1) terhadap semua jenis sel-sel kanser yang diuji, manakala gabungan RuPIP-NU1025 menghasilkan pelbagai kombinasi indeks; menunjukkan nilai CI antara sinergistik hingga antagonistik. Pertama sekali, daya hidup sel normal bagi semua gabungan ubat-ubatan yang dikaji adalah > 70%. Berdasarkan pengiraan purata nilai CI, gabungan sinergi (CI = 0.87) antara 25 µM RuPIP beserta 25 µM NU1025 atau 5 µM olaparib telah dipilih untuk ujian yang selanjutnya. Keupayaan sel untuk pulih dan membahagi dengan cepat untuk membentuk

koloni telah dikaji dengan menggunakan ujian klonogenik di mana rawatan sebatian sebagai agen tunggal menunjukkan kelangsungan hidup sel (S.F.) > 75%. Pertama sekali, gabungan RuPIP dan perencat PARP mengurangkan kelangsungan hidup sel di mana gabungan RuPIP-olaparib (S.F. < 2%) menunjukkan kesan pengurangan yang lebih ketara berbading RuPIP-NU1025 (S.F. < 57%). Di samping itu, rawatan tambahan dengan 25 μM RuPIP menyebabkan sel kanser menjadi sensitif terhadap rawatan olaparib di mana pengurangan nilai IC_{50} untuk olaparib bagi sel MCF7 adalah sebanyak 60 kali ganda (0.08 vs 4.75 μM), dan pengurangan nilai IC_{50} sebanyak > 300 kali ganda bagi sel MDA-MB-231 (0.06 vs 23.39 μM). Berikutnya, keupayaan sel untuk berhijrah telah dikaji dengan menggunakan ujian mimik luka yang menunjukkan bahawa rawatan dengan gabungan RuPIP-olaparib menyebabkan pengurangan keupayaan sel kanser untuk berhijrah yang ketara (P < 0.001) dengan pengurangan sebanyak 40% berbanding rawatan kawalan. Seterusnya, analsis aliran sitometri untuk menganalisis kitaran sel menunjukkan bahawa rawatan gabungan menyebabkan penangkapan kitaran sel pada fasa G1/S bagi sel A549 (17.1% pertambahan berbanding rawatan kawalan). Selain itu, kedua-dua sel MCF7 dan MDA-MB-231 menyebabkan penangkapan kitaran sel pada fasa G2/M (19.7% dan 20.4% pertambahan berbanding rawatan kawalan). Selanjutnya, ujian Annexin V-FITC menunjukkan bahawa rawatan gabungan menyebabkan peningkatan kematian sel yang ketara (P < 0.001) melalui mekanisme apotosis (25%, 30% and 31% pertambahan berbanding rawatan kawalan masing-masing bagi sel A549, MCF7 and MDA-MB-231) . Dan dapatan ini turut dikaitkan dengan peningkatan kerosakan DNA yang ketara (P < 0.001) apabila sel kanser dirawat dengan rawatan gabungan di mana terdapat pertambahan sel yang menghasilkan fokus γH2AX sebanyak 45% berbanding rawatan kawalan. Hasil dapatan kajian ini membuktikan bahawa RuPIP bersama perencat PARP dapat menghasilkan kesan sinergistik dalam pelbagai jenis sel kanser dengan kesan yang minimum terhadap sel normal.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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A549	Human lung epithelial carcinoma
ANOVA	One-way analysis of variance
BER	Base excision repair
BRCA	Breast cancer susceptibility gene
CD_3CN	Deuterated acetonitrile
$CDCl_3$	Deuterated chloroform
Chk1/2	Checkpoint kinases 1/2
CI	Combination index
DDR	DNA damage response
DIMS	Direct infusion mass spectrometry
Dm	Median-effect dose
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleotide
dppz	dipyrido[3,2-*a*:2′,3′-*c*]phenazine
DSB	Double-strand break
ESI-MS	Electrospray ionization mass spectrometry
Fa	Effect level (fraction affected)
FBS	Fetal bovine serum
FDA	U.S. food and drug administration
FTIR	Fourier transform infrared spectroscopy
HR	Homologous recombination
IC_{50}	Half maximal inhibitory concentration
Im	Imidazole
Kb	Binding constant
Log P	Octanol/water partition coefficient
m/z	Mass to charge ratio
MCF7	Human breast epithelial adenocarcinoma
MDA-MB-231	Human breast epithelial adenocarcinoma
MRC5	Normal human lung fibroblast
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NHDF	Normal human dermal fibroblast
NMR	Nuclear magnetic resonance
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PDT	Photodynamic therapy
phendione	1,10-phenantroline-5,6-dione
PI	Propidium iodide
PIP	2-(phenyl)imidazo[4,5-*f*][1,10]phenanthroline
RPC	Ruthenium polypyridyl complex
RuPIP	$[Ru(dppz)_2(PIP)]^{2+}$
S.F.	Survival fractions
SSB	Single-strand break
T24	Human urinary bladder epithelial carcinoma
TMS	Tetramethylsilane

CHAPTER 1

INTRODUCTION

1.1 Research background

Cancer remains as one of the primary causes of death associated with a high number of reported global incidences annually. Particularly, in 2018, 18.1 million new cancer occurrences and 9.6 million cancer-related mortality were reported, and importantly, these numbers are expected to rise within the next two decades (Bray *et al*., 2018; Siegel *et al*., 2019). Currently, the routine methods for cancer treatment are surgical resection or radiotherapy alongside periods of chemotherapy. However, the efficacy of these strategies is limited by various factors such as the mass of the tumor to be removed, the stage of tumor progression, the occurrence of metastatic tumors, the affordability of radiotherapy and the patient's health status (Abbas *et al*., 2018). Although recently, treatments using molecular targeted therapy have brought new hope, they are still not effective against advanced cancer and cancer recurrence despite initial high response rates (Saijo, 2012). Therefore, chemotherapy remains as the most common and realistic option for cancer treatment.

With the discovery of cisplatin (cis-diamminedichloroplatinum(II)), a platinum metal-based drug by Barnett Rosenberg in 1960, a milestone in the history of metal-based complexes in treating cancer was witnessed (Muggia *et al*., 2015). Cisplatin was the first metal-chemotherapeutic approved in 1978 by U.S. food and drug administration (FDA). Briefly, cisplatin induces inter- and intra-strands platinum-DNA crosslinks causing blockage of replication fork progression, leading to DNA damage in the form of cytotoxic DNA double-strand breaks (DSBs) (Deans, J. *et al*., 2013; Ndagi *et al*., 2017). However, cisplatin has limited clinical capability as it possesses inherent clinical drawbacks such as high general toxicity and less selectivity against healthy normal cells leading to severe adverse effects including myelosuppression, nephrotoxicity and neurotoxicity (Shaloam *et al*., 2014). Importantly, although effective in many other cases, the rapid occurrence of acquired or intrinsic resistance towards cisplatin presents as a well-established limit for its clinical application as these might significantly reduce their efficacy during treatment or even renders them ineffective leading to treatment failure and rapid progression of relapse (Eckstein, 2011; Amable, 2016). As such, these have encouraged substantial efforts in finding alternative anticancer agents based on other transition metals to replace current platinum-based drugs. Based on the success of cisplatin, inorganic medicinal chemists have since examined alternative transition metal centres such as ruthenium, palladium or rhodium, to design complexes which target highly proliferative cancerous cells with improved therapeutic windows compared to cisplatin (Ndagi *et al*., 2017).

Ruthenium metal-based complexes have attracted a great amount of interest in the last 20 years in combating cancer where majority of them owe their effects by forming coordinate interactions with DNA in a similar substitution kinetics to platinum complexes but differ in their reported biochemical properties and potency *in vitro* and *in vivo* (Ramadevi *et al*., 2015; Poynton *et al*., 2017). Within the last two decades, four ruthenium-based complexes have successfully entered clinical trials. The first ruthenium complex that entered clinical investigation was NAMI-A [ImH][*trans*-$RuCl_4$(DMSO)(Im)] (Im = imidazole, DMSO = dimethyl sulfoxide), followed by KP1019 [indazolium *trans*-tetrachlorobis(1H-indazole)ruthenate(III)] where both are proven to display anticancer properties with potent efficacy in clinics (Dyson *et al*., 2006). More recently, NKP1339, a derivative and more soluble sodium salt of KP1019 complex has demonstrated effective results in disease stabilization in the phase I study against solid tumors (Alessio *et al*., 2019). Encouragingly, these clinical findings demonstrated that these ruthenium complexes showed various clinical benefits compared to platinum drugs, including low general toxicity, greater tumor selectivity and potent efficacy on platinum-resistant tumors (Lin *et al*., 2018 a). Importantly, reduced severe adverse effects compared to platinum drugs were also noted.

Recently, polypyridyl complexes of Ru(II) (RPCs), a class of ruthenium complexes have emerged as promising drug candidates due to their ability to form non-covalent (reversible) interactions with DNA through intercalation of its organic ligand(s) between DNA base pairs (Zeglis *et al*., 2007; Gill *et al*., 2012). Most promisingly, the RPC photosensitizer TLD1443 $[Ru(dmb)_2(LL')]^{2+}$ where dmb = 4,4'-dimethyl-2,2'-bipyridine and LL' = 2-((2',2":5",2'"-terthiophene)-imidazo[4,5-*f*][1,10]phenanthroline) is currently undergoing phase II trials for bladder cancer patients (Lin *et al*., 2018 a). Our group has previously reported that the RPC, $[Ru(dppz)_2(PIP)]^{2+}$ where dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine and PIP = 2-(phenyl)imidazo[4,5-*f*][1,10]phenanthroline], or described as RuPIP hereafter binds to DNA through intercalation, leading to the stalling of DNA replication fork progression (Gill *et al*., 2016). In response to this DNA replication stress, the DNA damage response (DDR) signaling pathways are activated to counteract the effects of DNA damages and maintain the genomic integrity of cells.

Although RuPIP shows great anticancer properties, however, as in the case for the majority of anticancer drugs, treatment with a single agent may not lead to sufficient tumor suppression to improve disease outcome or patient survival (Basourakos *et al*., 2016; Mokhtari *et al*., 2017). If doses required as single agents for cancer cell killing are very high, this may even lead to intolerable toxicity to normal cells. Besides, for DNA targeting molecules, genotoxicity and even the generation of secondary cancers as a result of treatment are additional factors that must be considered. Recently, due to the enhanced understanding of cancer disease biology where several interconnected molecular pathways are involved in the system, using rational combination therapies of several drugs acting simultaneously on multiple targets and pathways are being sought to overcome the limited clinical options available within conventional chemotherapy (Chou, 2010; Foucquier *et al*., 2015). Synergistic combination chemotherapies

can lead to better efficacy, reduce drug dosage, decrease toxicity, and minimize risk of development of drug resistance and chance of relapse (Chou, 2006). Nowadays, drug combination therapies are common in clinical practices, and thus, using these therapeutic strategies to improve response to RuPIP would be a promising line of research.

DDR signaling pathways reverse the effects of DNA damage and are important in maintaining the stability of the human genome (Lu *et al*., 2018). Hence, the activity of these intrinsic DDR pathways can further predict treatment resistance and clinical outcomes in cancer therapy. Poly (ADP-ribose) polymerases (PARP) are one of the key DNA repair enzymes in DDR signaling pathways. Following DNA single-strand breaks (SSBs) or stalled replication forks, PARP mediates base excision repair (BER) pathway to prevent the generation of cytotoxic DSBs (Yang *et al*., 2004; Bryant *et al*., 2009; Beck *et al*., 2014). As PARP involves in DNA damage repair, the enzymatic inhibition of PARP leads to persistent stalling of replication fork progression and consequently, the formation of DSBs when fork collapse (Pascal, M., 2018). Therefore, PARPs have become the rational targets in anticancer drug research for the development of new drugs (Davar *et al*., 2012).

Several PARP inhibitors have progressed to clinical trials, and the PARP inhibitors olaparib (Lynparza®), niraparib (Zejula®) and rucaparib (Rubraca®) have been approved by FDA for treating cancers with defective breast cancer susceptibility gene (BRCA). However, while improved therapeutic response to PARP inhibition in BRCA1/2 mutated-cancers has been shown, PARP inhibitor exerts limited efficacy in the treatment of other cancers without homologous recombination (HR) protein or gene defect (Basourakos *et al*., 2016). The role of the metallo-intercalator RuPIP in the activation of DDR signaling has attracted our interest in combining RuPIP with PARP inhibitors to effectively inhibit repair of damaged DNA and achieve synergism in cancer cells. This will enhance the efficacy of RuPIP and ultimately, improve cancer cell killing while reducing the cytotoxic effect on normal cells. In addition to this, examining the combination of PARP inhibitors alongside RuPIP will expand the use of PARP inhibitors to a wider cancer population including BRCA wild type cancer.

1.2 Problem statement

As in the case for the majority of anticancer drugs, treatment with single agent or monotherapy may not lead to sufficient tumor suppression to improve disease outcome or patient survival. In addition to this, if doses required as single agents for cancer cell killing are very high, this may lead to intolerable toxicity to normal cells resulting in severe adverse effects. Typically, for DNA targeting molecules, genotoxicity and even the generation of secondary cancers as a result of treatment are additional factors that must be considered. Moreover, although BRCA1/2-deficient cancers demonstrate exquisite sensitivity to PARP inhibitors when used as single agents, they represent a relatively small subset of cancer patients which limits PARP inhibitors' clinical capabilities. As such, this rational combination approach of RuPIP and PARP inhibitor will expand the clinical use of PARP inhibitors to a greater cancer population. The synergistic activities will result in better efficacy, decreased dosage, reduced toxicity, reduced severe adverse effects and are more effective in limiting the emergence of drug resistance. Consequently, this rational combination therapies have potential in achieving sufficient tumor suppression, and ultimately, improving disease outcomes and patient survival.

1.3 Research objectives

The aim of this study is to determine the effect of RuPIP in combination with PARP inhibitors NU1025 or olaparib in various human cancer cells as a new therapeutic strategy and explore the mechanistic basis of synergy. In this study, finding proof of significant superiority of the effect of combination treatment in comparison to single agent condition is of particular importance.

i. To determine the cytotoxic effects of RuPIP or PARP inhibitors (NU1025, olaparib) as single agents on four different cancer cell lines (A549, MDA-MB-231, MCF7 and T24) and on normal human fibroblast cell lines (NHDF, MRC5).
ii. To determine the potential synergism of RuPIP in combination with PARP inhibitor against the cancer cell lines.
iii. To determine the potential cytotoxicity mechanisms underlying the synergistic combination of RuPIP and PARP inhibitor using cell cycle analysis, Annexin V-FITC apoptosis assay and through quantification of double-strand break (DSB) DNA damage.

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LIST OF PUBLICATIONS

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