p53-MDM2 INTERACTION TARGETED THERAPY BY NUTLIN-3 ON NASOPHARYNGEAL CARCINOMA CELLS

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ABSTRACT

Nutlin-3, a small-molecule inhibitor of p53-Mdm2 interaction, is known to be effective against cancers expressing wild-type (wt) p53. p53 mutations are rare in nasopharyngeal carcinoma (NPC), and hence targeting the disruption of p53-Mdm2 interaction to reactivate p53 may offer a promising therapeutic strategy for NPC. This study hypothesized that reactivation of p53 in NPC cells may suppress NPC cell proliferation, and in addition, Nutlin-3 combined with cisplatin may further suppress the cancer cell proliferation more effectively. To investigate these possibilities, the effects of Nutlin-3 alone or in combination with cisplatin were tested on C666-1, an Epstein-Barr virus (EBV)-positive NPC cell line bearing wt p53, in parallel with normal nasopharyngeal epithelial (NPE) NP69 and NP460 cells. Single drug treatment resulted in a concentration-dependent inhibitory effect on the cancer cell proliferation. Cisplatin was more cytotoxic to the NPE cells compared to the NPC cells, while Nutlin-3 was more effective and selective in inhibiting NPC cells. Cisplatin combined with Nutlin-3 showed stronger anti-proliferative effect against NPC cells and markedly suppressed its anchorage-independent growth on soft agar, suggesting that combined treatment was more effective than single drug therapy. Prior verification showed that C666-1, NP69 and NP460 cells retained the wt p53. Treatment with Nutlin-3 showed significant accumulation of p53, p21Waf1/Cip1 and Mdm2 proteins in cells expressing wt p53 in comparison to p53-mutated cells. The effect of Nutlin-3 on the restoration of p53, p21Waf1/Cip1 and Mdm2 expression was impaired following p53-knocked down in NPC cells, and likewise the cells with p53 knockdown showed less sensitivity to Nutlin-3. These findings suggest that Nutlin-3 activates the p53 pathway and exerts its cytotoxicity on NPC cells in a p53-dependent manner. The accumulation of Annexin V/PI-stained cells showed treatment of NPC cells with cisplatin resulted in apoptosis and Nutlin-3-treated cells showed less percentage of apoptotic cells compared to the

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cisplatin-treated cells. Apoptosis, however, increased significantly in the cells treated with cisplatin and Nutlin-3. Similarly, Nutlin-3 positively upregulated BAX and PUMA protein expressions in NPC cells. The expression levels of these proteins also increased significantly in cells treated with cisplatin and Nutlin-3, concomitant with the detection of cleaved PARP level. Taken together, these findings suggest that Nutlin-3 sensitises NPC cells to cisplatin-induced apoptosis by modulating pro-apoptotic targets via the p53 pathway. In addition, an extended treatment period of NPC cells with Nutlin-3 did not result in the emergence of p53-mutated cells, albeit reduced sensitivity to Nutlin-3 was observed. This stresses on the importance of treatment duration and clinical doses optimization to improve the efficacy of Nutlin-3 significantly. Therefore, the overall findings revealed supportive evidence of the effectiveness of combining cisplatin with Nutlin-3 as potential therapy against NPC.

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ABSTRAK

Nutlin-3, suatu molekul kecil yang merencat interaksi p53-Mdm2, dijangka berkesan menentang sel kanser yang mewarisi gen p53 berciri liar (wt) tanpa mutasi. Mutasi p53 jarang berlaku pada karsinoma nasofarinks (NPC), maka pengaktifan p53 melalui gangguan interaksi p53-Mdm2 merupakan suatu strategi terapeutik yang berpotensi untuk merawat NPC. Kajian ini menyarankan hipotesis bahawa pengaktifan semula p53 dalam sel NPC menyekat proliferasi sel NPC; dan gabungan Nutlin-3 dengan cisplatin berupaya menyekat proliferasi sel kanser dengan lebih berkesan. Bagi menyiasat kemusykilan ini, kesan Nutlin-3 tunggal atau digabung dengan cisplatin telah diuji ke atas C666-1, sel NPC yang membawa infeksi virus Epstein-Barr (EBV) dan mengekspresikan wt p53. Ujian dilakukan seiring dengan sel normal epitelial nasofarinks (NPE) NP69 dan NP460. Rawatan dengan ubat tunggal merencat proliferasi sel kanser berkadar kepada dos. Cisplatin lebih sitotoksik terhadap sel NPE daripada sel NPC, manakala Nutlin-3 adalah lebih efektif dan selektif terhadap perencatan sel NPC. Gandingan cisplatin dengan Nutlin-3 menghasilkan kesan anti-proliferatif lebih kuat terhadap sel NPE berbanding sel NPC, dan merencat pertumbuhan bebas-tambatan (*anchorage-independent*) sel dalam agar lembut dengan lebih ketara. Pemerhatian ini menyarankan bahawa rawatan kombinasi adalah lebih berkesan daripada rawatan tunggal yang digunakan secara berasingan. Sel C666-1, NP69 dan NP460 telah disahkan mengekspresikan wt p53. Rawatan Nutlin-3 menghasilkan pengekspresan protein p53, p21Waf1/Cip1 dan Mdm2 secara kumulatif dalam sel wt p53 berbanding dengan sel p53-bermutasi. Kesan Nutlin-3 memulihkan pengekspresan p53, p21Waf1/Cip1 dan Mdm2 yang terjejas berikutan penyenyapan p53 dalam sel NPC; pada masa yang sama, sel dengan penyenyapan p53 adalah kurang sensitif kepada Nutlin-3. Hasil kajian ini menyarankan bahawa Nutlin-3 mengaktifkan laluan p53 dan menghasilkan kesan sitotoksik terhadap sel NPC secara pergantungan-p53. Kehadiran

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sel positif-pencelup Annexin V/PI menunjukkan cisplatin mengaruh apoptosis dalam sel NPC, manakala sel yang dirawat dengan Nutlin-3 menunjukkan peratusan sel apoptotik rendah berbanding dengan sel dirawat cisplatin. Apoptosis meningkat dalam sel dirawat cisplatin dan Nutlin-3 secara bererti. Nutlin-3 mengatur pengekspresan protein BAX dan PUMA dalam sel NPC. Tahap pengekspresan protein-protein ini turut meningkat secara signifikan, seiring dengan pengekspresan PARP yang dikesan dalam sel dirawat cisplatin dan Nutlin-3. Kesimpulannya, hasil kajian mencadangkan bahawa Nutlin-3 memekakan sel NPC kepada apoptosis yang diaruh cisplatin secara memodulasikan sasaran pro-apoptotik melalui laluan p53. Di samping itu, rawatan lanjutan Nutlin-3 ke atas sel NPC bagi jangka masa panjang tidak mengaruh pembentukan p53-bermutasi, meskipun mencetus sel untuk menjadi kurang sensitif kepada Nutlin-3. Ini menegaskan bahawa optimasi tempoh rawatan dan dos klinikal adalah penting bagi menambahbaikkan keberkesanan Nutlin-3. Bolehlah disimpulkan bahawa keputusan kami menyokong keberkesanan rawatan kombinasi cisplatin dan Nutlin-3 sebagai regimen rawatan yang berpotensi untuk merawat NPC.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
^0C	Degree Celsius
α	Alpha
µg/mL	Microgram per millilitre
µl	Microlitre
µm	Micrometre
µM	Micro molar
APS	Ammonium persulfate
APAF-1	Apoptotic protease-activating factor 1
ASR	Age-standardized incidence rate
ATCC	American tissue culture collection
ATM	Ataxia-telangiectasia mutated
ß	Beta
BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X Protein
BID	BH3 interacting domain death agonist
Bcl2	B-cell lymphoma 2
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
cm	Centimetre
CH3	Methyl group
CO_2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
dH_20	Distilled water
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunoasorbant assay
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	DL-dithiothreitol
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
e.g.	For example

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ELISA	Enzyme-linked immunosorbent assay
et al.	'And other people whose names are not mentioned'
FCS	Foetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
g	Gram
GC	Guanine–Cytosine
GFP	Green fluorescence protein
h	Hour(s)
H_0	Null hypothesis
H_1	Alternative hypothesis
H_2O	Water
H_2O_2	Hydrogen peroxide
Hdm2	Human double minute-2
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid hemisodium salt
HRP	Horse radish peroxidase
IC_{50}	Half maximal inhibitory concentration
kDa	Kilo Dalton
L	Litre(s)
Lenti-shΔp53	p53 knockdown by lentiviral-based small-hairpin RNA
M	Molar
Mcl-1	Myeloid cell leukaemia 1
Mdm2	Murine double minute-2
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar(s)
mt-p53	Mutant p53
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW	Molecular weight
NaCl	Sodium chloride
NCR	National Cancer Registry
NFM	Non-fat dry milk
nm	Nanometre(s)
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1

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NPC	Nasopharyngeal carcinoma
OD	Optical density
p53	Protein 53
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PES	Phenazine ethosulfate
pH	Measure of the acidity /basicity of a solution
PI	Propidium iodide
PMSF	phenylmethylsulfonylfluoride
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RRM2B	Ribonucleotide Reductase M2 B
RT	Room temperature
sec	Second(s)
SD	Standard deviation
SDS	Sodium dodecyl sulphate
ST13	Suppression of Tumorigenicity 13
T_a	Annealing temperature
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline tween-20
TEMED	N,N,N,N'-tetramethylenendiamine
T_m	Melting temperature
Tris-Cl / Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
TRITC	Tetramethyl rhodamine iso-thiocyanate
Tween 20	Poly-oxyethylenesorbitanmonolaureate
UV	Ultra-violet
V	Volt
WHO	World Health Organization
wt	Wild-type

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CHAPTER 1: INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a common epithelial squamous cell head and neck carcinoma, which originates from the nasopharyngeal mucosa layering the upper part of the throat. NPC is strongly associated with Epstein-Barr virus (EBV) infection (Chai *et al.*, 2012) and intake of salted fish (Armstrong and Chan, 1983). The aetiology of NPC is multifactorial, including smoking (Schleper, 1989), occupational exposures (Hildesheim *et al.*, 2001) and genetic susceptibility (Heo *et al.*, 1989). NPC is fairly rare in most parts of the world, indicating distinct racial and geographical distribution. NPC is common in North Africa, the Middle East, Greenland, Taiwan and especially Southern China (Devi *et al.*, 2004; Zeng and Zeng, 2010). NPC is prevalent among the natives of Southeast Asia including Malaysia. In Malaysia, NPC is the fourth most frequent cause of cancer mortality, and is the third most common in males (Zainal and NorSaleha, 2011). NPC is highly prevalent among the Bidayuh from East Malaysia, followed by the local native Chinese and Malays; Indians rarely have NPC (Khoo and Pua, 2013; Pua *et al.*, 2008).

NPC is commonly treated with radiotherapy and/or chemotherapy based on the different stages of the disease (Lee *et al.*, 2012b; Zhang *et al.*, 2013); surgery is rarely the main therapeutic option for NPC due to its complex anatomical proximity to critical structures. In an effort to improve the prognosis and efficacy of NPC therapy, a combination of definitive radiotherapy plus cisplatin-based chemotherapy is recommended (Lee *et al.*, 2002). Concurrent chemo-radiotherapy is the main treatment modality for advanced-stage NPC (Zhang *et al.*, 2013). The most active chemotherapy agents used in NPC are cisplatin and 5-fluorouracil (5-FU) (Lee *et al.*, 2012b; Zhang *et al.*, 2013). These drugs are highly toxic and carry the risk of damaging the surrounding tissues and organs (Anniko and Sobin, 1986; Choi *et al.*, 2015). In addition, early stage

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NPC may be asymptomatic or can present with apparently trivial symptoms, thus likely to be ignored, which results in diagnosis delay and subsequently, treatment failure (Khoo and Pua, 2013; Pua *et al.*, 2008). Late stage NPC is associated with poor prognosis and treatment failures (Chan *et al.*, 2004; Zhang *et al.*, 2013). In fact, recurrence, distant metastases, resistance and adverse effects of treatments remain as major challenges in the clinical scenario (Phua *et al.*, 2013; Tuan *et al.*, 2012). Therefore, reducing undesirable complications of chemotherapy drugs is a major goal in pharmaceutical research for NPC treatment.

p53, a tumour suppressor gene, is often overexpressed in cancer cells. p53 is a transcription factor which controls the genes involved in DNA repair, cell cycle arrest and apoptosis (Brown *et al.*, 2009). Targeting the activation of p53 pathway plays a central role in preventing cancer development by inducing growth arrest, apoptosis, senescence and angiogenesis. Nutlins are cis-imidazoline analogues (Vassilev *et al.*, 2004) which compete with murine double minute-2 (Mdm2) for binding to p53. Nutlin-3 has been reported to be effective in killing cancer cells expressing wild-type (wt) p53 (Kastan, 2007; Vassilev, 2007). Nutlin-3 exerts anti-cancer effects on acute myeloid (Kojima *et al.*, 2005) and chronic lymphocytic leukaemia (Saddler *et al.*, 2008), multiple myeloma (Stuhmer *et al.*, 2005), Kaposi sarcoma (Ye *et al.*, 2012), liposarcoma (Müller *et al.*, 2007), rhabdomyosarcoma (Miyachi *et al.*, 2009), Ewing's sarcoma (Sonnemann *et al.*, 2011), colon (Hori *et al.*, 2010) and testicular cancers (Koster *et al.*, 2011), osteosarcoma and other types of cancers (Tovar *et al.*, 2006). Nutlin-3 suppressed not only tumour growth, but also distant metastasis in a xenograft model of wt p53 neuroblastoma (Van Maerken *et al.*, 2009). Moreover, Nutlin-3 selectively enhances apoptosis in wt p53 cancer cells by activating the p53 pathway (Kojima *et al.*, 2005; Van Maerken *et al.*, 2009).

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Currently, agents that reactivate the p53 pathway are undergoing clinical trials (Khoo *et al.*, 2014). Although Nutlin-3 has been reported to be effective against a wide variety of tumour bearing wt p53, the effects of Nutlin-3 on NPC cells have yet to be reported. p53 mutations have been reported to be rare in NPC (Effert *et al.*, 1992; Hoe *et al.*, 2009), even in recurrent radioresistant NPC (Chang *et al.*, 2002), thus making this type of cancer a potential candidate for treatment with p53-Mdm2 inhibitors, like Nutlin-3. Interestingly, it has been suggested that the overexpression of p53 using an adenoviral vector was effective against NPC cells (Pan *et al.*, 2009; Weinrib *et al.*, 2001) indicating that further increasing p53 levels by using the p53 activator, Nutlin-3 may be effective to further improve NPC treatment.

The present study sought to investigate the effects of Nutlin-3 alone or in combination with cisplatin on C666-1, an EBV-positive NPC cell line bearing wt p53 and two other normal nasopharyngeal epithelial (NPE) cell lines, NP69 and NP460. This study also tested whether extended treatment with Nutlin-3 could result in the emergence of p53 mutations in NPC cells. The findings of the present study may provide further insights on the potential use of Nutlin-3 as a new treatment in the arsenal against NPC.

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CHAPTER 2: LITERATURE REVIEW

2.1 Human Nasopharyngeal Carcinoma (NPC)

2.1.1 The Biology and Histological Subtypes of NPC

Squamous cell carcinoma which originates from multiple sites of the upper aerodigestive tract is classified as head and neck cancer. Head and neck cancers are heterogeneous, which include oral, oropharyngeal, laryngeal, nasal cavity and paranasal sinus, nasopharyngeal, hypopharyngeal, salivary gland and thyroid cancers. The pharynx is made up of nasopharynx, oropharynx and hypopharynx (laryngopharynx) (Figure 2.1) (Vokes *et al.*, 1993). The nasopharynx, a narrow tubular portion of the upper part of the passage behind the nasal cavity, connects the back of the nose to the back of the mouth. Nasopharyngeal tumour arises from the nasopharynx. The most common type of nasopharyngeal tumour is NPC, which is a unique malignant epithelial carcinoma of the head and neck region (American Cancer Society, 2013; Roland and Paleri, 2011). NPC has a common pattern of spread and is often related to the neck lymph nodes as its primary site.

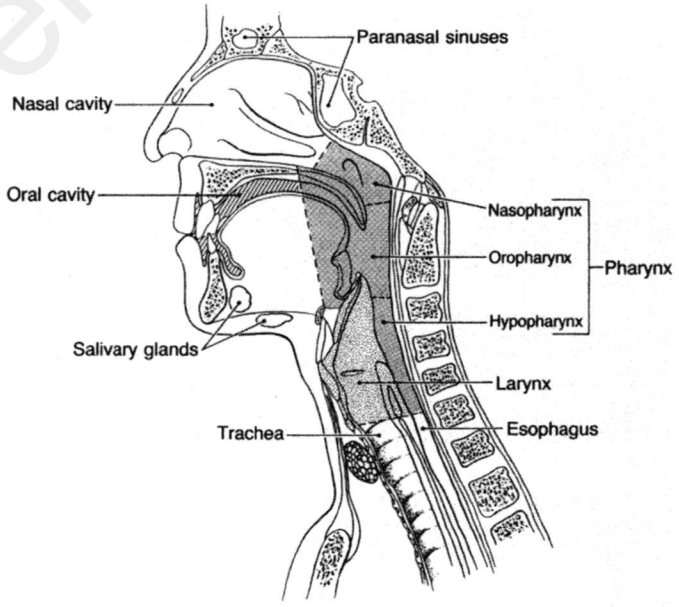

Figure 2.1: Schematic diagram showing the sagittal section of the upper aerodigestive tract (Vokes *et al.*, 1993).

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Nasopharyngeal tissue consists of several types of cells; each type has the ability to transform into different types of tumours. These differences are significant for the classification of the disease subtype, severity as well as the efficacy of treatment. The nasopharynx disease arises from nasopharyngeal epithelial cells which transforms and propagates uncontrollably, forming a nasopharyngeal tumour. The disease has been diagnosed and classified since the early 20th century. Initially, it was coined as "the base skull cancer" by Michaux in 1845 (Wei *et al.*, 2011), but was then histologically classified into three groups by Citelli and Calamida in 1903 (Nicholls and Niedobitek, 2013).

In the early 1900s, NPC histopathology was further analysed and classified as "endothelioma", "lymphoepithelial carcinoma" or "transitional cell carcinoma" by Trotter (1911), Reverchon *et al.* (1921) and Quick and Culter (1927), respectively (Wei *et al.*, 2011). The classification was further subdivided into five types by Ewing in 1929 (Wei *et al.*, 2011). The pathology of NPC was re-considered based on the simplified and the detailed classification. The classification was based on the biological behaviour, variation in morphology, degree of differentiation as well as the clinical and prognosis of the tumours. The international WHO classification was first proposed by Shanmugaratnam and Sobin (1978) and was corrected by European pathologist (1991) (Wei *et al.*, 2011). Currently, NPC is classified into three major types based on the degree of differentiation. Based on classification WHO I, keratinising squamous cell carcinoma is similar to other head and neck cancer; while the non-keratinising (differentiated) squamous carcinoma and undifferentiated carcinoma is classified as WHO II and III, respectively (Nicholls and Niedobitek, 2013; Wei *et al.*, 2011). The WHO I tumour cells are seen in 5–10% of NPC cases; they display squamous differentiation with the presence of intercellular bridges and the production of either

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intracellular or extracellular keratin. The WHO II tumour cells show pavement design or stratified arrangement where individual cells are clearly separated by cell margins. The WHO III tumour cells appear as a syncytial sheet, with a mass containing round vesicular nuclei and prominent nucleoli (Pathmanathan *et al.*, 1995). The 1991 WHO classification scheme was then updated in 2005 be more comprehensive for the determination of treatment options and prognosis (Nicholls and Niedobitek, 2013; Wei *et al.*, 2011).

2.1.2 Epidemiology of NPC Worldwide

Although it is considered as rare, NPC has claimed tens of thousands lives worldwide with a remarkable geographic distribution. NPC incidence remain significantly high in endemic regions of Southern China (Yu and Yuan, 2002), Hong Kong, Taiwan, Northern Africa (Roland and Paleri, 2011) and Southeast Asia, with a high prevalence among the Malaysian Bidayuh and the Chinese (Khoo and Pua, 2013; Pua *et al.*, 2008). According to the global scale statistics for the year 2012 (GLOBOCAN, 2012), NPC is the 23rd most common cancer with nearly 80,000 new cases diagnosed annually and a mortality rate that exceeds 50,000. NPC is more prevalent in males than females, with the male to female ratio of 2.3:1.0 (Parkin *et al.*, 2001). High-risk rates of ASR 25–30 (ASR expressed as cases per 100,000 populations) were seen in Southern China notably among the Cantonese in the area of Guangzhou; high incidence rates are also noted in Southeast Asia. In contrast, low incidence rates of ASR < 1 were observed in Europe and North America (GLOBOCAN, 2012).

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2.1.3 Epidemiology of NPC in Malaysia

According to the National Cancer Registry Report (2007) (Zainal and NorSaleha, 2011), NPC was the fourth most common of overall cancers in Malaysia. NPC was reported to be the third most frequent cancer in males and the eleventh among females. The incidence of NPC increases with age, predominantly between the ages of 40–55 years. In Peninsular Malaysia, NPC is more prevalent among Chinese males than Malays and Indians; males are four times more likely to develop NPC compared to females. NPC rarely affects the Indians (Zainal and NorSaleha, 2011). According to the Sarawak Cancer Registry 1996-2000 , NPC was the second most common cancer in Sarawak, afflicting largely patients between the age of 45 and 54 years old (Zainal and NorSaleha, 2011). The incidence of NPC is reported to be extremely high among the native Bidayuh, which recorded the highest ASR of 31.5 with 2.3-fold (males) and 1.9-fold (females) higher risk than the mean Sarawak ASR (Devi *et al.*, 2004). Clinically, 80% of the total patients diagnosed in the Sarawak General Hospital originate from Kuching and Serian (Tiong and Selva, 2005). In 2007, new NPC cases diagnosed in Sarawak was seemed to be the most common cancer among males (15.8%) and the fifth most common cancer in females (5.8%) (Zainal and NorSaleha, 2011). It is estimated that the incidence of NPC in Malaysia is growing rapidly with 2447 new cases expected to be diagnosed in year 2020 (GLOBOCAN, 2012).

2.1.4 Aetiology and Pathogenesis of NPC

NPC has an unusual racial distribution which is strongly associated with multifactorial aetiologies, such as the intake of demethylnitrosamine-contaminated salted fish (Roland and Paleri, 2011), environmental risk factors, cigarette smoking (Schleper, 1989), occupational exposures (Hildesheim *et al.*, 2001), gamma herpes EBV infection (Chai *et al.*, 2012) and genetic susceptibility (Lo *et al.*, 2012; Roland and

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Paleri, 2011). Alcohol consumption, a common lifestyle in the West and other parts of the world, has been identified as another important risk factor for the development of NPC (Cheng *et al.*, 1999).

NPC is more prevalent among the Chinese populations which may be attributed to their lifestyles, such as consumption of large amount of carcinogen-contaminated salted fish. A case-control study suggested consumption of Cantonese-style salted fish has a strong correlation with NPC (Yu *et al.*, 1986). In addition, early childhood exposure to diet that is high in preserved foods and salted fish is shown to have significant effects on higher NPC risk (Yu and Yuan, 2002; Zheng *et al.*, 1994). Approximately 90% Hong Kong (Yu *et al.*, 1986), 60% Malaysian Chinese (Amstrong & Chan, 1983) and 50% Guangzhou (Yu *et al.*, 1989) NPC cases are attributed to frequent consumption of salted fish in childhood. Several case-control studies observed that high-risk NPC populations frequently have high intake of preserved food, pickled vegetables and fermented products, such as beans, bean pastes, eggs and seafood pastes (Lee *et al.*, 1994; Yuan *et al.*, 2000).

Salted fish is rich in the carcinogenic volatile nitrosamine, an EBV activating agent, which is accounted as an NPC-causing agent. Nitrosamine metabolising genes, cytochrome CYP2E1 and CYP2A6 are responsible for NPC susceptibility (Hildesheim *et al.*, 2001; Tiwawech *et al.*, 2006). A study conducted among Chinese populations revealed that consumption of Chinese medicine is another dietary-related factor for NPC. A significant correlation between traditional herbal medicine consumption and increased NPC risk has been linked with NPC pathogenesis (Gallicchio *et al.*, 2006). Several commonly used herbal plant extracts have the ability to reactivate EBV as well

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as increase the titres of anti-EBV antibody in EBV-infected host (Hildesheim *et al.*, 2001).

In 1966, Old *et al.* discovered that NPC is an EBV-associated cancer, especially the most common WHO types II and III NPC (Old *et al.*, 1966). EBV is a gammaherpesvirus consists of an icosahedral capsid bearing a double stranded DNA core for the expression of approximately 100 genes. The presence of monoclonal episome (Raab-Traub, 2002) and expression of viral genomes (Pathmanathan *et al.*, 1995) have been detected *in situ* of NPC cases. Almost 95% of NPC tumours are EBV associated (Chou *et al.*, 2008).

EBV infection is classified as EBV Latency I, II and III. EBV infects normal resting B cells and induces virus transformation into lymphoblastoid cells through coordinated expression of six nuclear proteins (EBNA 1, 2, 3A, 3B, 3C and LP) and three latent membrane proteins (LMP 1, 2A and 2B) (Eliopoulos *et al.*, 1999). NPC is characterised by the transcription of EBV-encoded small nuclear RNAs (EBERs), which encode EBV nuclear antigens EBNA-1, LMP-1 and LMP-2. The expression of these nuclear antigens is found in EBV latency II, and is the characteristic of many EBV-associated tumours such as Hodgkin disease, T-cell non-Hodgkin lymphoma and gastric carcinoma. NPC is consistently associated with EBV infection (95%), and were shown to overexpress p53 (95%), LMP-1 (85%), Bcl-2 (80%) and co-expression of LMP-1 and Bcl-2 (95%) (Sheu *et al.*, 2004).

NPC has tumorigenic potential due to the unique activity of EBV latent genes of EBNA-1, EBNA-2, LMP-1 and LMP-2. EBNA-1 is a transcriptional activator capable of developing viral DNA partitioning during replication, while EBNA-2 is the major

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transcriptional regulator of EBV latency gene expression (Raab-Traub, 2002). EBNA-1 expression in NPC enables viral episome to segregate with the host chromosomes during mitosis, and must be expressed to enable the viral genome to be transmitted to the daughter cells. EBNA-2 initiates and modifies the transcription of target genes, which eventually governs the activation of resting B-cell, cell cycle entry and proliferation of the growth transformed cells (Raab-Traub, 2002).

LMP-1 is the most important oncoprotein in EBV-related malignancies, which is postulated to be involved in the development of NPC (Dawson *et al.*, 2012). LMP-1 as a viral "transforming" gene inhibits epithelial cell growth and differentiation, induces morphologic transformation, as well as engages in signalling pathways for the regulation of diverse cellular functions such as proliferation or apoptosis. LMP-1 expression is essential for EBV-induced B-cell immortalization *in vitro*, prevents cell death through the up-regulation of the anti-apoptotic genes Bcl-2, Bcl-xL, Mcl-1 and A20 (Eliopoulos *et al.*, 1999). The expression of LMP-1 also results in phenotypic changes, cytokine production and differentiation blockade in epithelial cells, a property which is responsible for the pathogenesis of NPC. LMP-1 expression in NPC indicates a role for EBV oncogene in the early stages of pathogenesis (Dawson *et al.*, 2012). One well-defined function of LMP-1 that contributes to its oncogenic properties is its ability to protect epithelial cells against apoptosis (Dawson *et al.*, 2012).

Early studies have suggested that the overexpression of p53, Mdm2 and Bcl2 is commonly detected in NPC. Indeed, a recent study showed that LMP-1 modulated the p53-dependent transcriptional activities and G_1-S cell-cycle checkpoint, repressed DNA repair and initiated tumourigenicity in NPC cells (Liu *et al.*, 2004). In additional, LMP-1-expressing cells have a tendency to activate oncogenic pathways, which suppress the

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activation of p53 pathway (Yang *et al.*, 2014). LMP-1 was shown to have ability in enhancing DNA-damage-induced micronuclei formation related to chromosomal aberration, and reduced the cellular capacity for DNA repair in a p53-independent manner (Liu *et al.*, 2004; Dawson *et al.*, 2012). Although LMP-1 does not induce anti-apoptotic gene Bcl2 in epithelial cells, it can modulate p53 activity via stimulates Mdm2 expression, which promotes p53 turnover (Wu *et al.*, 2004). LMP-1 contributes to the development of NPC through the repression of p53-dependent transcriptional activity (Yang *et al*, 2014), as well as synergises with Bcl-2 to inhibit growth suppression induces by wt p53 in NPC cells (Sheu *et al.*, 2004). Hence, suggesting that LMP-1 can also synergise with mutant p53 to provide a growth advantage to the initiation and progression of NPC (Sheu *et al.*, 2004).

Ataxia-telangiectasia mutated (ATM) is essential for the initiation of signalling in DNA damage response in epithelial cells. The activation of ATM leads to cell cycle arrest, DNA repair and apoptosis. Constitutive expression of LMP-1 enhanced the radiosensitivity of NPC cells through suppressing ATM expression (Yang *et al*, 2014). The inactivation of the p53-mediated apoptosis pathway may contribute to the resistance of LMP-1-induced NPC cells to apoptosis; this event could be linked to tumour recurrence in post-radiotherapy patients (Yang *et al.*, 2014). When expressed in tumourigenic epithelial cells, LMP-1 potentiates anchorage-independent growth and promotes cell motility, invasion, metastasis and angiogenesis (Dawson *et al.*, 2012). In addition to LMP-1, LMP-2 exerts profound effects on a variety of cellular processes include mediation of tumour cell proliferation, survival and migration (Chou *et al.*, 2008). A recent study showed that LMP-2A expression in NPC contributed to conserve EBV latency (Dawson *et al.*, 2012).

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The majority of NPC tumours contain intact p53 genes (Effert *et al.*, 1992). A significant association was also established between the overexpression of wt p53 and EBV infection in NPC. EBV-infected tumours were found more likely to express higher level of p53 than tumours lacking EBV, suggesting that EBV can interact with p53 and contribute to its overexpression (Gulley *et al.*, 1998). However, there is evidence of the overexpression of p53 in EBV-positive NPC tumours in the absence of alterations in the p53 gene suggested that the EBV presence and p53 overexpression and mutations are not correlated (Hoe *et al.*, 2009; Nasrin *et al.*, 1994).

Aside from these reported events, EBV infection also plays an important role in the aetiology of NPC. The severity of EBV infection varies with carcinoma type, with nonkeratinizing type II and III carcinomas having the highest titers of IgA and IgG antibodies to EBV (Raab-Traub, 2002). These antibodies, which frequently herald ahead the appearance of the tumour, serve as prognostic biomarkers of treatment response, remission and relapse. EBV statuses have prognostic implications, where plasma EBV DNA load is another useful monitoring tool for NPC. Pre-treatment and post-radiotherapy plasma EBV DNA levels have an excellent correlation with treatment response, with high sensitivity (96%) and specificity (93%) (Lee *et al.*, 2012b). Several recent studies suggested that the levels of EBV DNA were persistently low in patients with remission, while higher in patients with relapsed NPC (Razak *et al.*, 2010). Patients with elevated pre-treatment EBV DNA levels corresponded to decreased disease-free survival and increased risk of recurrence or disease-related death (Razak *et al.*, 2010); and increased post-treatment EBV DNA levels is associated with high risk of tumour recurrence (Zhang *et al.* 2013). Therefore, EBV DNA in the post-treatment plasma of NPC patients correlated significantly with the tumour load, and accurately predicts the recurrence and surveillance (Yang *et al.*, 2014; Lee *et al.*, 2012b).

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Nasopharynx is located at the top of the throat, which connects the mouth and nose to the oesophagus and larynx, and is a part of the respiratory tract. Epidemiological studies suggest that higher NPC risk from exposure of nasopharynx tissue to toxic pollutants in the air, (e.g. wood dust, micro-particles of cigarette smoking and chemical carcinogens) is biologically plausible (Hildesheim *et al.*, 2001). Cigarette smoking contributes to moderate effect of NPC risk among the Taiwanese, Philippines and Southern China populations (Chen *et al.*, 1990; West *et al.*, 1993; Yu and Yuan, 2002). Another study suggested that approximately 60% of type I NPC, but not types II and III, can be attributed to cigarette smoking (Vaughan *et al.*, 1996). Exposure to aromatic hydrocarbon smoke by burning incense or anti-mosquito coils has been postulated as another important risk factor for NPC (West *et al.*, 1993). Extended exposure to wood dust, smoke, formaldehyde and certain aromatic hydrocarbons has been determined as a serious concern in contributing to NPC risk (Hildesheim *et al.*, 2001; Yu and Yuan, 2002).

The findings of genome-wide studies confirmed that NPC oncogenesis is strongly related to multiple genetic alterations, which involved chromosomal (allelic imbalances of 3p, 9p, 11q, 12q, 13q, 14q, and 16q), genetic (gene amplification, deletion and mutation) and epigenetic (methylation) factors (Hui *et al.*, 1999; Lo *et al.*, 2012; Lo and Huang, 2002). All the factors contribute to the development of NPC by disrupting cell proliferation and differentiation, affecting genome stability and the expression of apoptotic genes. An array-based comparative genomic hybridization study demonstrated that chromosomal gains in 3q27.3–28, 8q21–24, 11q13.1–13.3 and 12q13, with oncogene cyclin CCND1 over-expression, are associated with the development of NPC (Hui *et al.*, 2005). Several case-control studies revealed NPC susceptibility with polymorphisms of CYP2E1, GSTM1, XRCC1 and hOGG1 genes. Lu and colleagues

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(Lu *et al.*, 2003) suggested that genes associated with susceptibility to NPC are also located within the HLA-A locus. The deletion of tumour suppressor genes (ADAMTS9 and LRIG1) at chromosome 3p12.3–p14.2; and the gain of genes (GPR160 and SKIL) at 3q26.2–q26.32 are significant as genomic markers for NPC prognosis (Sheu *et al.*, 2009).

Using a combination of whole-exome sequencing, targeted deep sequencing and single-nucleotide polymorphism (SNP) array analysis, Lin and colleagues recently determined the first genome-wide view of the mutational landscape of NPC associated with clinical significance (Lin *et al.*, 2014). The results revealed nine significantly mutated genes with the two most commonly been found in NPC were PIK3CA and p53, and the seven newly identified were BAP1, ERBB2, ERBB3, KRAS, MLL2, NRAS and TSHZ3. Aside from these reported events, the integrated analysis identified multiple recurrent copy number variations affecting several important cellular processes and pathways including chromatin modification, ERBB-PI3K signalling and autophagy machinery, many of which had not been previously implicated in NPC.

The SNP array analysis revealed that chromatin-modification pathway as among the most frequently altered pathways in NPC, with ARID1A being the most frequently altered gene in the chromatin-modification pathway (Lin *et al.*, 2014). The ARIDIA gene was reported to inhibit cell proliferation through regulating p21 gene expression in NPC. Hence, the loss of ARIDIA gene in NPC significantly increased anchorage-independent colony formation, cell migration and xenograft growth, as well as is strongly associated with EBV burden and poor overall survival (Lin *et al.*, 2014). In addition to ARID1A mutation, EBV-positive tumours had frequent BAP1 mutations. ARID1A and BAP1 genes encode tumour suppressors, were found frequently lost in

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NPC suggesting that p53 mutations are common in metastasis and advanced stages disease (Lin *et al.*, 2014). In a comparative study using several previously sequenced tumours, p53 is the most frequently mutated gene in epithelial malignancies (Lin *et al.*, 2014). However, now with the most sensitive next-generation whole-exome sequencing approach, the data indicated that NPC results in a relatively low level of genomic alteration, as well as rare p53 mutation frequency of <10% were observed in EBV-associated NPCs (Lin *et al.*, 2014).

2.1.5 Clinical Symptoms of NPC

NPC is also categorised based on its early and late symptoms. The majority of NPC cases are asymptomatic with apparently vague symptoms in the early stage. However, the appearance of painless neck lumps as the first sign of NPC is reported in almost 50% of newly diagnosed NPC patients. Symptoms include a persistent bloody nasal discharge and headaches during the early stages of NPC (Prasad and Pua, 2000; Suzina and Hamzah, 2003). Postnasal dribbling or nasal obstruction occurs when the tumour enlarges and obstructs the air passage via the nasopharynx.

The late stages may show ear symptoms, such as feeling like the ears are blocked which is caused by the accumulation of fluid in the middle ear, as well as Eustachian tube obstruction which may cause pain in the ear and give rise to deafness. The late symptoms include double vision, facial pain and headache (Barnes *et al.*, 2005; Prasad and Pua, 2000). The most unfortunate aspect is asymptomatic undetectable metastasis with the tumour growing relentlessly to a visible size before its manifestation (Barnes *et al.*, 2005; Prasad and Pua, 2000).

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2.2 Diagnosis, Treatment and Challenges of NPC

2.2.1 Diagnosis and Treatment Options

Diagnosing NPC commonly begins with a physical examination, serological test for immunoglobulin A against EBV and nasal endoscopy. Suspected cases with confirmed diagnosis, elevated anti-EBV titres, or suspicious nasal endoscopic findings, will undergo staging based on the nasopharyngeal tissue biopsies and imaging examinations. Computerised tomography (CT scan), magnetic resonance imaging (MRI) and positron emission tomography (PET) confirm the diagnosis, delineate the tumour's size, disease extent and metastasis distance (Zhang *et al.*, 2013). The sensitivity and specificity of the endoscopic examination and CT scan were reported to be 75%, 94.3% and 50%, 49.1%, respectively (Chao *et al.*, 2003). The endoscopic biopsy, nasopharyngeal endoscopy and CT scan yielded a sensitivity of 83.3%, 66.6% and 50%; but a specificity of 100%, 95% and 45%, respectively (Ragab *et al.*, 2008); suggesting that biopsy and endoscopy have higher specificity in NPC diagnosis when compared to CT scan. The retropharyngeal lymph node metastasis determined by MRI, CT scan and PET-CT was 45.3%, 39.6% and 20.8%, respectively; indicating that MRI is the most significant test when compared to CT scan and PET-CT scan (Su *et al.*, 2006).

Determination of NPC's stage and histological grade is useful for determining the severity and predicting the extent of the cancer spread. The stages of NPC are defined based on the TNM system, which ranges from stages I to IV (Zhang *et al.*, 2013). TNM is an abbreviation for tumour (**T**), node (**N**) and metastasis (**M**). NPC stages are determined based on the size and location of the primary tumour (**T**); the effect of tumour to the lymph nodes (**N**) and the effect of tumour to other parts of the body (**M**) (Zhang *et al.*, 2013). The stages, together with patient's history and past illness as well as treatments are considered when determining the type and effectiveness of the

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treatment and prognosis. Treatment options such as surgery, radiotherapy (RT), chemotherapy (CT) or combined chemo-radiotherapy (CCT) are dependent on the specific site(s) and stages of the tumour.

2.2.1.1 Radiation Therapy (RT)

RT is commonly used to treat non-metastatic early-stage NPC (Zhang *et al.*, 2013), which improved the 5-year survival rates of stages I and II NPC (Heng *et al.*, 1999). Intensity-modulated (IMRT) and external beam RT (EBRT) are used for eliminating the primary site of untreated NPC (Mould and Tai, 2002; Wei and Sham, 2005).

Brachytherapy is an ionising internal RT generated by the implantation of a temporary or permanent radioactive device directly inside or adjacent to the tumour (Shigematsu *et al.*, 1983). Brachytherapy damages tumour in a smaller area and at a shorter time compared to EBRT. Brachytherapy alone or in combination with EBRT, CT or surgery improved cancer cure rate and reduced the adverse effect of CT (Mould and Tai, 2002).

Stereotactic radiosurgery (SRS), a non-surgical 3D computerised RT (Leksell, 1983) that irradiates tumour with minimal exposure to normal tissue, was developed to treat invasive and locally recurrent NPC (Suarez *et al.*, 2010; Xiao and Xu, 2010).

2.2.1.2 Chemotherapy (CT)

CT utilises cytotoxic agents to shrink large and firm tumours, and to kill or stop the growth of tumour cells. CT prolonged symptom-free survival mainly in asymptomatic patients presented with metastasis (Wee *et al.*, 2005). Cisplatin, [*cis*-$PtCl_2(NH_3)_2$] or CDDP is one of the most widely used platinum-containing CT agent to treat solid

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tumours as well as NPC. The platinum complex triggers apoptosis by causing crosslinks to the guanine bases on the DNA (Jamieson and Lippard, 1999).

5-Fluorouracil (5-FU) is one of the oldest antineoplastic CT agents known for treating solid tumours, as well as head and neck cancers (Chau and Cunningham, 2002; Li *et al.*, 2004; Tebbutt *et al.*, 2002). 5-FU is a p53-dependent cell cycle specific antimetabolic agent which triggers apoptosis by inducing DNA damage and can halt the specific phases of cell cycle (Qin *et al.*, 2008; Wee *et al.*, 2005).

When compared to other head and neck cancers, NPC is more sensitive to the combination of cisplatin and 5-FU. However, combinations of 5-FU, platinums, anthracyclines, gemcitabine, methotrexate and taxanes are associated with higher complication rate which typically involve normal tissue toxicity. CT is delivered via bloodstream to overcome the limitation of RT in the deepest regions, significantly controlled metastasis of chemo-radiosensitive NPC (Wee *et al.*, 2005). CT followed by RT enhanced the effectiveness of RT and improved the efficacy of subsequent treatments (Ma *et al.*, 2001). CT given after RT further eliminated the radiated cancerous NPC cells (Chi *et al.*, 2002). The administration of cisplatin-5-FU followed by RT significantly improved the 5-year and 7-year disease-free survival rates (Ma *et al.*, 2001; Ma *et al.*, 2002). Concurrent CCT reduced distant failure and improved the survival rate of locoregionally advanced NPC (Chang *et al.*, 2000).

2.2.1.3 Surgery

Surgery is rarely offered as an important therapy for NPC because the affected site is deep seated in the centre of the head, a technically challenging area to operate on (Wilson, 1951). Surgery is reserved for failure to treat recurrent local tumours following

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primary RT or/and CT especially if the tumour is located in an operable site (Chan, 2014), as well as to remove the fragmented NPC tissues and prevent metastasis (Lou *et al.*, 2014; Wang *et al.*, 2015). Surgery improved the salvage rates of local failure-free and disease-free survival in recurrent T1 and T2 NPC (Lee *et al.*, 2012a). However, there are arguments against these traditional approaches because surgery is an invasive procedure, limited accessibility of the nasopharynx and high risk of disfiguration. Hence, minimally invasive endoscopic techniques were developed in the early 2000s to minimise the risk of traditional surgical complications (Mai *et al.*, 2009; Monteiro and Witterick, 2014; Tay *et al.*, 2014). Endoscopic nasopharyngectomy is reserved in salvaging local recurrence of NPC following radio-chemotherapy which has not metastasised. Endoscopy alone or in combination with robotic surgery is an effective treatment modality for recurrent T1, T2 and T3 NPC (Monteiro and Witterick, 2014; Tay *et al.*, 2014). Endoscopic surgery yielded better outcomes than open surgery, salvaged T3/4 NPC, lowered the complication rates and improved the quality of life (Na'ara *et al.*, 2014).

2.2.1.4 Targeted Immunotherapy

Epidermal growth factor receptor (EGFR) is over-expressed in solid malignant tumours, mainly in the head and neck, and colorectal cancers. The expression of EGFR in NPC is correlated with increased risk of locoregional failure and reduced survival of advanced NPC (Chua *et al.*, 2004). The anti-EGFR, cetuximab, is the first monoclonal antibody specifically designed to inhibit EGFR in both normal and cancer cells. KRAS gene encodes K-Ras protein for regulating cell division and the effect of cetuximab is wt KRAS-dependent. The binding of cetuximab to EGFR induces cell-cycle arrest and apoptosis as well as turning off the metastasis and angiogenesis. Cetuximab enhances the sensitivity of cancer cells to radio-chemotherapy. It has been approved by the FDA

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(March 2006) for use during CT with cisplatin or along with RT (Bou-Assaly and Mukherji, 2010; Martinelli *et al.*, 2009).

2.2.2 Complications of NPC Therapies

The selection of treatment option is dependent on the tumour location and disease extent (Huang *et al.*, 2012). Early stage NPC is asymptomatic; this often delayed diagnosis until stages III/IV of the disease. Hence, NPC patients tend to experience therapy failure and have poor prognosis. The 5-year overall survival rate of stages III and IV NPC patients was reported to be 56.2% for RT and 47.2% for CT (Leung *et al.*, 2005). Local recurrence is one of the major therapy failure patterns (Chan *et al.*, 2004). NPC patients are often adversely affected by post-radio-chemotherapy effects because radiation and chemotherapy do not differentiate between normal and cancerous cells.

The major concerns of RT are specific organ damage in the head and neck regions due to exposure to harmful radiation. The damaging effects of radiation on components of the auditory system have been reported since 1905 by Ewald. RT contributed to radiation-induced sensory neural hearing loss (SNHL) with an incidence rate of 24.2% (Kwong *et al.*, 1996). IMRT improved the locoregional control of NPC, but distant metastasis and recurrence remained as the main treatment failure (Lee *et al.*, 2012a; 2012b). Moreover, advanced stage NPC has been shown to have poor prognosis when treated with IMRT (Tham *et al.*, 2009).

CT is not cancer specific and damages actively dividing cells during mitotic phase (Chan *et al.*, 2012). Cisplatin is extensively used mainly due to its effectiveness in treating difficult to cure cancer, but is also associated with nephrotoxicity, neurotoxicity, ototoxicity, alopecia, myelotoxicity, nausea and vomiting (Anniko and

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Sobin, 1986; Chitapanarux *et al.*, 2007; Kwong *et al.*, 1996). Among the severe adverse effects to 5-FU are chest pain associated with heart disease, fatigue, nausea, mouth sores, photophobia (light sensitivity) and diarrhoea.

The use of CT either before or after RT for patients with advanced NPC has shown to have no improvement on the overall survival or relapse-free survival (Chi *et al.*, 2002; Ma *et al.*, 2001). Concurrent advanced CCT technique is considered to be effective for patients with early stage NPC. However, nearly 50% NPC patients experience cancer recurrence; 80% reported cases of incurring auxiliary late complications of long-term toxicity effect (Phua *et al.*, 2013) and 43% treated-patients showing distant metastases within four years (Lee *et al.*, 2002; Sultanem *et al.*, 2000). The emergence of chemo-resistance remains as major obstacle for cisplatin-based CT which impaired the cisplatin-induced apoptosis (Siddik, 2003). The addition of cisplatin to CCT increased the risk of SNHL, severe hearing impairment or deafness (Kwong *et al.*, 1996).

Traditional surgery leads to transposition of obstructing bone and soft tissue, damages to the nerves and blood vessels, weakness in the arm or lower lip and numbness of the ear (Mould and Tai, 2002; Tay *et al.*, 2014). Endoscopic nasopharyngectomy causes significant postoperative complications including surgical pain, headache, nasopharyngeal necrosis, and crusting of the otitis media (Mai *et al.*, 2009; Monteiro and Witterick, 2014). Cetuximab therapy rarely has side effect, but patients may develop severe skin allergy, infusion reaction, anaemia, cardiac toxicity and lung disease (Bou-Assaly and Mukherji, 2010).

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2.3 p53 and Mdm2 Interaction as a Drug Targeted Therapy

2.3.1 Tumour Suppressor Gene p53 and its Important Roles

In 1979, an unknown 53 kDa molecular mass protein was first discovered as a transformation-associated 53,000-Dalton (53K) protein by two independent groups of scientists (Lane, 1992; Linzer and Levine, 1979). The p53 gene is a tumour suppressor gene and has been termed as the "guardian of the genome" (Lane, 1992), or "cellular gatekeeper of growth and division" (Levine, 1997) due to its important biological role in the G_1-S checkpoint, preventing gene aberration for genome stability by eradicating DNA-damaged cells (Lane, 1992). Tumour suppressing activities of the wt p53 protein was recognised in human cancers (Finlay *et al.*, 1989) and Mills confirmed that wt p53 contributes tumour-suppressive capabilities compared to mutated p53, which is oncogenic (Mills, 2006).

The p53 gene is located on chromosome 17 (17p13.1). The wt p53 phosphoprotein contains 11 exons which encode for a 2.8 kb mRNA, translated into a 53 kDa protein made up of 393 amino acids (Harlow *et al.*, 1985; Matlashewski *et al.*, 1984). It has three major functional domains; the N-terminal transcription-activation domain (TAD), central core DNA-binding domain (DBD) for sequence-specific DNA binding and C-terminal tetramerisation domain (OD) (Figure 2.2) (Bai and Zhu, 2006). The 1st exon is a region for a stable stem-loop structure and is recognised as a non-coding region. The stem-loop structure is bound to wt p53, but not to mutant p53. There are two untranslated regions (UTR) found located between the 1st, 2nd and 11th exons. The tetramerisation and transcription-activation domains are important to facilitate DNA and Mdm2 binding, respectively. The presence of a zinc atom and two-beta sheets in the DNA-binding domain further stabilises the p53-DNA complex (Cho *et al.*, 1994).

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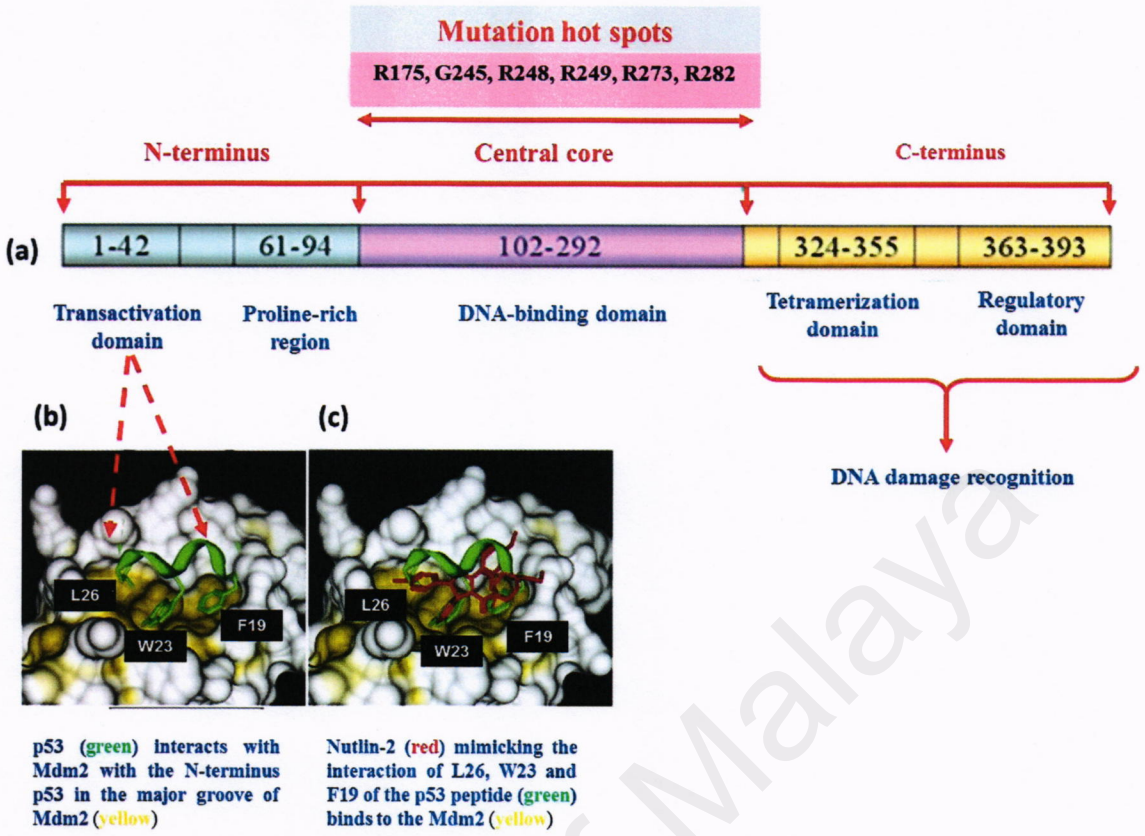

Figure 2.2: Schematic representation for (**a**) p53 gene structure and mutation hot spots; (**b**) The interaction of p53-Mdm2 at the p53's transactivation domain of N-terminus. The p53-amino residues (**green**) Leu-26 (L26), Trp-23 (W23) and Phe-19 (F19) are essential for binding in the major groove of Mdm2 (**yellow**); (**c**) Structural model revealed that Nutlin-3 could mimic the orientation of these three amino residues, fill the p53-binding pocket, disrupt the p53-Mdm2 interaction and liberate functional p53 (Bai and Zhu, 2006; Vassilev, 2007).

p53 is a potent pro-apoptotic protein with a short half-life of 6–20 minutes. The degradation of p53 is inhibited in cellular stress, resulting in prolonged p53 half-life to hours and the accumulation of p53 in the cell (Harris and Levine, 2005). As a DNA damage-inducible nuclear transcription factor, p53 determines whether the damaged cells are repaired or to self-destruct (Saito *et al.*, 2002). Upon irreparable cellular damage, p53 induces apoptosis through p53-mediated intrinsic or extrinsic apoptotic pathway. In response to irreparable DNA damage, p53 activates mainly the intrinsic apoptotic pathways. The intrinsic apoptosis is dominated by pro-apoptotic BAX and Bcl-2 proteins involving the release of cytochrome C from mitochondria to cytoplasm,

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resulting in activation of caspase. The extrinsic pathway is associated with death receptors that induce caspase activation, leading to apoptotic cell death (Haupt *et al.*, 2003). p53 initiates the gene expression for pro-apoptotic (BAX, BID, NOXA, PUMA, APAF-1) and anti-apoptotic (Bcl2, Mdm2) genes. The reactivation of p53 triggers DNA repair (RRM2B, ST13), cell cycle arrest (p21) and senescence or cell death by apoptosis (BAX, PUMA), thereby protecting the cell from malignant transformation (Table 2.1, Figure 2.3) (Brown *et al.*, 2009).

The wt p53 is essential for protecting cells from genome mutation or becoming cancerous by regulating the transition of G_1-S phase in renewable functional tissue, suppresses tumour development and eradicates tumour in a natural way. Similar outputs were reported in human tumour xenografts (Brady and Attardi, 2010; Kastan, 2007). A genomic landscape study showed G_1-S cell cycle transition was affected in 28% of human NPCs, with most frequent deletion occurs in 9p21 and p53 (Lin *et al.*, 2014). The ability of the p53 gene to prevent cancer can be reduced, impaired or lost to mutagens, pathogens or lesions. Mutations in p53 alter its ability for DNA repair, thus contributing to cancer development. p53 is one of the most commonly mutated gene in early stage of human lung, colon, head and neck cancers (Figure 2.4). Mutant p53 has a long half-life when compared to wt p53 found in over 60% of head and neck cancer. p53 mutations have been reported to be rare in NPC (Effert *et al.*, 1992; Heo *et al.*, 1989). The mutation is rare even in recurrent radiotherapy refractory NPC (Chang *et al.*, 2002). Point mutations of p53 gene detected in hot spots of exons 5, 7 and 8 are less than 10% of NPC (Sun *et al.*, 1992). However, increased staining of p53 protein by immunohistochemistry has been reported (Chen *et al.*, 1995; Hoe *et al.*, 2009), suggesting that gene alterations are present either in the p53 or the downstream pathways (Hoe *et al.*, 2009; Hui *et al.*, 1999; Zhang *et al.*, 2014).

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Table 2.1: Functional history of p53 in somatic cells

p53 target genes	Regulation of p53-mediated pathway
DRAM	Autophagy
ICAM-1	Innate immunity
LIF	Embryo implantation
Mdm2	p53 regulation
p21	Cell cycle arrest
PAI-1	Senescence
PUMA	Apoptosis
R2	DNA repair
SCD2	Metabolism
TIGAR	Inhibition of ROS*/survival
TSP1	Inhibition of angiogenesis

*ROS: Reactive oxygen species

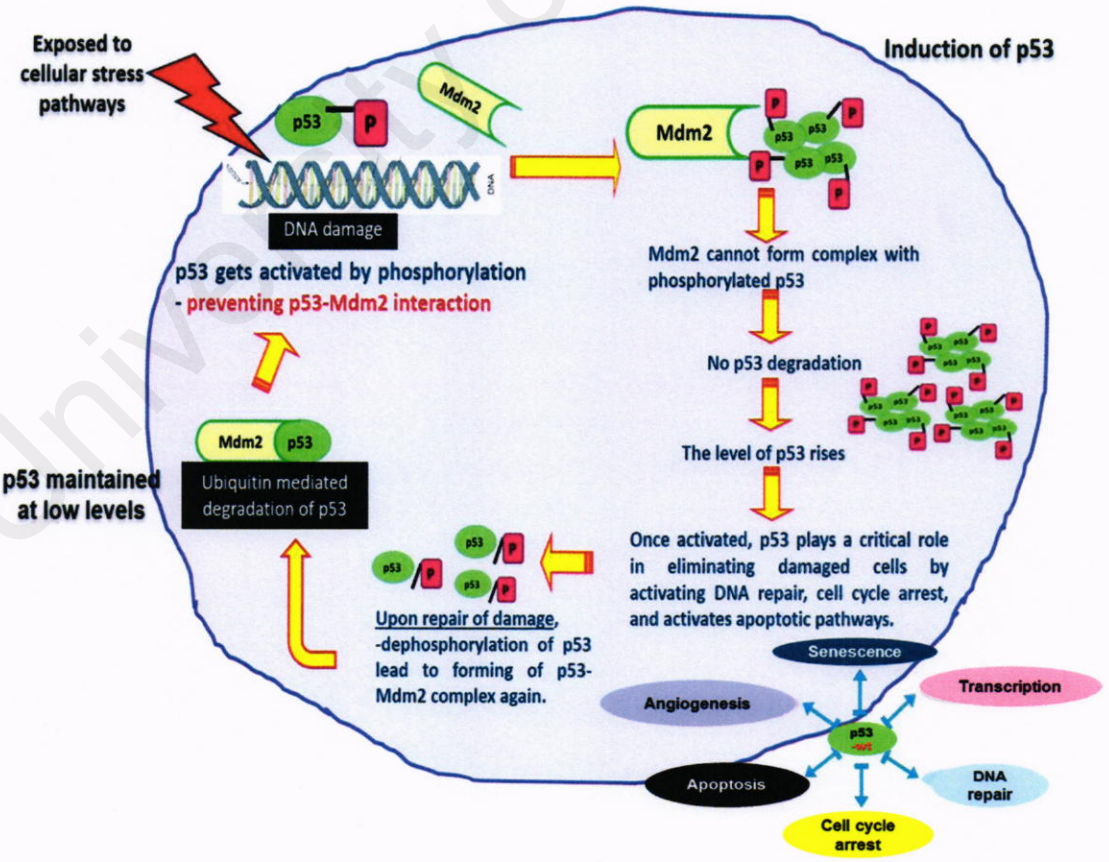

Figure 2.3: p53 induces cell cycle arrest, DNA repair and apoptosis (Michael and Oren, 2003; Midgley and Lane, 1997; Vassilev *et al.*, 2004).

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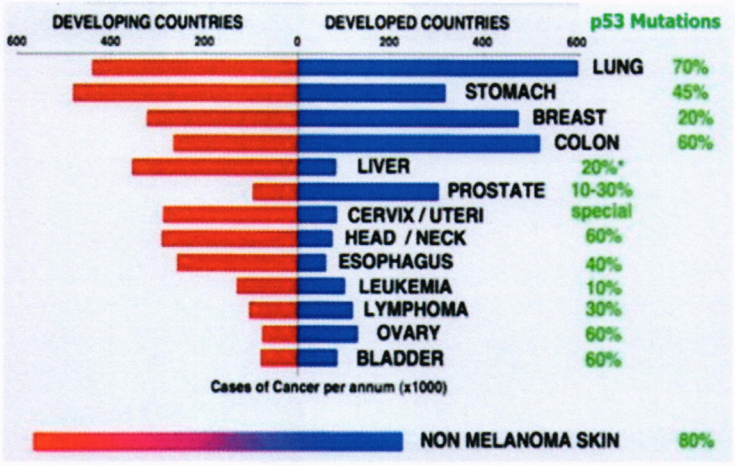

Figure 2.4: Worldwide distribution of cancers and p53 mutations (Soussi, 2012). Adapted from The TP53 web site *http://p53.free.fr/p53_info/p53_cancer*

The downstream targets mediated by p53 play a major role for cell cycle arrest, apoptosis and genomic stability. Inactivation of p53 leads to an increase of mutation frequency resulting from inefficient nucleotide excision repair. Imperfect nucleotide excision repair causes genomic stability, which is manifested as gene amplification and chromosomal aberrations. This indicates that the loss of p53 function promotes nucleotide alterations, which disable transcription of p53 protein or its downstream mediators. These events have been detected in the 4^{th} to 9^{th} exons, and clusters at residues 100–300 coding region DNA binding domain (DBD). Genetically altered concentrations of conserved DBD region in exons 5^{th} to 8^{th} were identified in 90% of p53 mutations; where missense mutations were detected in 78% (Hainaut *et al.*, 1997). Thirty percent of the alteration was clustered in the hot spots of codons 175, 245, 248, 249, 273 and 282 (Hainaut *et al.*, 1997; Olivier *et al.*, 2002). The prevalence of p53 alteration in human cancers was determined at 13%, 67%, 70% and 87% as deletion/insertion, single base substitutions (T>G), (A>G), (G>T), respectively (Greenblatt *et al.*, 1994).

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Mutant p53 is genetically unstable, produces defective protein with major conformational changes, which disables DNA binding and loses the ability for regulating p53-mediated proteins (Bunz *et al.*, 1999; He *et al.*, 2002; Shiao *et al.*, 1994). The loss of functional p53 allele is associated with an increased risk of developing Li-Fraumeni syndrome in early adulthood (Olivier *et al.*, 2002). Deletion of p53 co-activator, ARF gene, results in dysfunction of p53 target operating protein (Sherr, 1998). Mutation leads to p53 gain-of-cancer-promoting activities, including the establishment of tumour invasion by affecting integrin and EGFR (Ko and Prives, 1996; Muller *et al.*, 2009).

2.3.2 Oncogene Mdm2

Oncogene is a term for a gene that, when mutated or expressed at abnormally high level, can cause a normal cell to become a cancerous cell. The murine double minute 2 (Mdm2), known in human as Hdm2, is an oncogene located on the acentromeric extra chromosomal nuclear bodies of chromosome 12 named double minutes (12q15). Mdm2 gene encompasses 12 exons which encode for a 491-amino acid long phosphoprotein. Mdm2 interacts with its negative regulator proteins such as p53, retinoblastoma (Rb), E2F1 and p14ARF. In a normal cell, p53 binds to a specific region on Mdm2 in a hydrophobic cleft structured by the Mdm2-amino terminal involving three p53-amino residues transactivation domain Leu-26, Trp-23 and Phe-19 (Figure 2.5). Mdm2 protein is the main regulator in mastering the stability and activity of p53 protein (Schon *et al.*, 2002; Vassilev, 2007).

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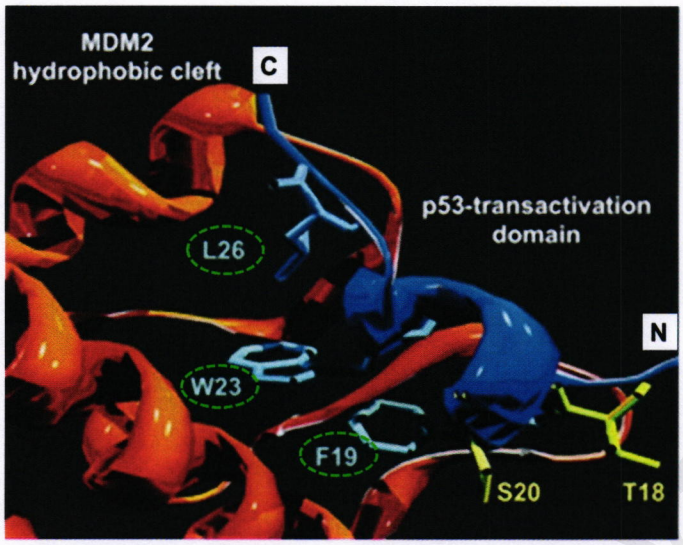

Figure 2.5: X-ray crystallography, capture of the p53-Mdm2 complex. This structure demonstrates a hydrophobic cleft which shows the binding of p53 (**dark blue**) to the groove of Mdm2 (**orange**). The binding is established by only three side chains of p53-amino residues Leu-26 (**L26**), Trp-23 (**W23**) and Phe-19 (**F19**) (Schon *et al.*, 2002).

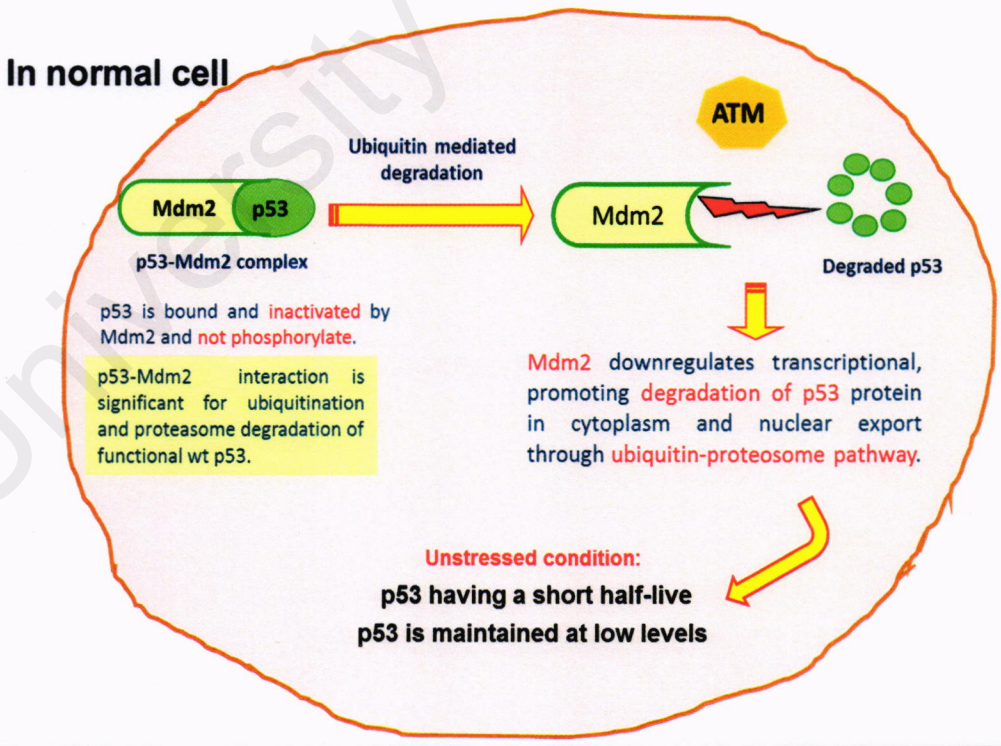

Figure 2.6: The stability of p53 in normal cell. The regulation of p53 is tightly monitored by its oncogenic E3 ubiquitin ligase negative regulator Mdm2 (Michael and Oren, 2003; Midgley and Lane, 1997; Vassilev *et al.*, 2004).

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2.3.3 Interaction of p53-Mdm2

The low basal level of p53 is constantly regulated by continuous ubiquitylation and 26S proteasome degradation via three regulatory actions: (1) Direct inhibitory binding of p53 to Mdm2: p53 acts as a transcription factor for Mdm2 and promotes self-inhibition as part of the negative regulatory loop (Harris and Levine, 2005); (2) Degradation of ubiquitinated-p53 by ubiquitin ligase action of Mdm2: Mdm2 acts as an oncogenic E3 ubiquitin ligase negative regulator for ubiquitylation and proteasome degradation of functional p53, which is necessary for protein stabilisation, activation and retention within the cytoplasm; and, (3) Nuclear export of p53 to cytoplasm: Mdm2 represses transcriptional p53 protein via Mdm2-mediated ubiquitin-proteasome pathway, nuclear export and degradation in cytoplasm (Figure 2.6) (Mirzayans *et al.*, 2012).

In the absence of stress, p53 is almost undetectable in proliferating cells which retards inappropriate growth arrest and apoptosis. Mdm2 as a p53 interacting protein tightly regulates p53 by Mdm2 E3-ubiquitin ligase activity. Activation of p53 enhances the stimulation of protein kinase on conformational change in p53 protein, thus interfering with the p53-Mdm2 interaction. Dissociation of p53-Mdm2 complex in response to cellular stress, DNA damage or activation of oncogene leads to p53 activation (Appella and Anderson, 2001). p53 protein is triggered, stabilised and released from Mdm2 binding, resulting in accumulation of p53.

2.3.4 Reactivation of p53 by Nutlin-3 for Human Cancer Therapy

The development of small molecular weight protein-protein interaction inhibitors has been reported to target the repression on Mdm2 to restore the function of p53 tumour suppression. In 2004, Vassilev and colleagues discovered a class of small molecular

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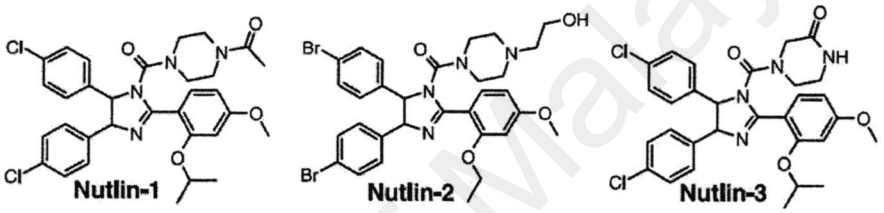
weight compounds capable of blocking the p53-Mdm2 interaction. These compounds were named after the town of Nutley, where the research was conducted, as nutlins (Vassilev *et al.*, 2004). The conformation, hydrophobicity and imidazoline backbone of these compounds were designed by mimicking the p53 binding pocket. These series of non-peptide *cis*-imidazoline analogues consist of Nutlin-1, Nutlin-2 and Nutlin-3 (Figure 2.7) which can disrupt the p53-Mdm2 interaction, compete with p53 for Mdm2 binding and displace p53 from its complex with Mdm2.



R1	Cl	Br	Cl
R2	H	H	O
R3	COH	$(CH_2)_2OH$	H
R4	CH_3	H	CH_3

Figure 2.7: The chemical structure of Nutlins inhibitors with their respective functional groups. R, a generic alkyl group.

Nutlin-1 induces the up-regulation of Mdm2 and p21, as well as cell cycle arrest in G_1 and G_2 phases. Nutlin-3 ($C_{30}H_{30}Cl_2N_4O_4$, 581.4896 g/mol) is effective and non-genotoxic in stabilising p53 in experimental models (Vassilev, 2007). Firstly, Nutlin-3 dominated p53 binding site on the hydrophobic groove of Mdm2 and promoted the dissociation of p53; secondly it abolished the negative inhibitory effect of Mdm2 by antagonising the p53-Mdm2 interaction for p53 breakdown and finally salvaged and protected p53 from ubiquitin-mediated proteasomal degradation leading to restoration of p53 (Kojima *et al.*, 2005; Shangary and Wang, 2009; Van Maerken *et al.*, 2009).

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Since its discovery, *in vitro* and *in vivo* therapeutic-based studies have revealed the antitumour activity of Nutlin-3 as a potential alternative targeted therapy for the current chemotherapy regime. Nutlin-3 has been reported to induce the regulation and activation of p53 pathway in tumours expressing wt p53 (Vassilev *et al.*, 2004). Nutlin-3 induced apoptotic cell death in leukaemia (Kojima *et al.*, 2005; Saddler *et al.*, 2008), multiple myeloma (Stuhmer *et al.*, 2005), osteosarcoma, melanoma, prostate, colorectal (Tovar *et al.*, 2006), colon (Hori *et al.*, 2010) and testicular cancers (Koster *et al.*, 2011). Nutlin-3 is non-genotoxic and protects normal cells against mitotic toxicity (Apontes *et al.*, 2011; van Leeuwen and Lain, 2011) and kidney cells from the cytotoxic effect of cisplatin (Jiang *et al.*, 2007). Nutlin-3 suppresses not only tumour growth but also distant metastasis in a xenograft model of wt p53 neuroblastoma (Van Maerken *et al.*, 2009).

The National Comprehensive Cancer Network (NCCN) clinical practice guidelines recommended utilisation of multiple approaches to improve the prognosis of hardly curable NPC. Despite the high initial response and successful local control rates achieved with advance RT and/or CT in stage III and IV NPC, NPC remains incurable with unacceptable 5- and 10-year survival rates (Leung *et al.*, 2005). CT administered concomitantly with RT had no benefit for overall survival or relapse-free survival (Chi *et al.*, 2002; Ma *et al.*, 2001). Severe radio-chemo adverse effects, late diagnosis, complications, therapy-resistance, loco-regional recurrence and distant metastases remain as major causes of mortality and morbidity in NPC (Phua *et al.*, 2013; Razak *et al.*, 2010; Tuan *et al.*, 2012). In view of these issues and challenges, the design of modern cancer therapeutic must evolve from non-specific targeting that affects both normal and cancer cells to specifically targeting unique molecular signature of cancer

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cells and to produce greater antitumour efficacy with less toxicity effects, such as p53-based targeted therapy.

This study proposed NPC is a potential candidate for p53-based targeted therapy with Nutlin-3 due to the low frequency of p53 mutations in this tumour (Momand *et al.*, 1998; Lin *et al.*, 2014). Cell culture studies were utilised to demonstrate that the tumour suppressive activity of p53 can be regained, causing NPC cells to undergo apoptosis. However, there is a limited availability of *in vitro* established-NPC cell lines. EBV infection has been shown to have a critical role in NPCs aetiology; particularly the WHO type III undifferentiated NPC. The latent gene of EBV, such as LMP-1 which is known to be expressed in NPC, has been reported to inhibit the p53 pathway (Liu *et al.*, 2004). Most of the NPC cell lines used today were established 10 to 30 years ago, the majority of which are EBV negative. These cell lines which include CNE1, CNE2, HONE1, AdAH and NPC-KT were once EBV positive but the EBV genome was lost due to prolonged subculture (Dittmer *et al.*, 2008). Furthermore, these cell lines are not unique NPC cell lines as they are positive for human papillomavirus 18 (HPV-18) and are cross-contaminated with HeLa cells (Strong *et al.*, 2014).

At present, C15, C17, C18 and C666-1 are the only available EBV-positive NPC cell lines. The C15, C17 and C18 are transplantable NPC tumour lines that require propagation by subcutaneous passage into nude mice (Busson *et al.*, 1988), unlike the C666-1 cells which can be propagated by *in vitro* culture. C666-1, an undifferentiated NPC cell line, stably retains wt p53, unlike HK1 and C17 which harbour mutated and deleted p53, respectively. Moreover, C666-1 serves as an ideal investigative tool in the present study as it consistently harbours a natural EBV infection in long-term culture (Cheung *et al.*, 1999). These characteristics would allow observation of cell growth

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inhibition and p53 pathway activation in EBV latent genes-positive C666-1 cells. To our knowledge no study has previously reported the effects of Nutlins on this cell line. Our main research answer is to develop proof-of-concept p53-based targeted therapy which could provide further insights into the important role played by Nutlin-3 to further improve the treatment of NPC; then we could cure NPC.

2.4 Objectives of the Study

This study aimed to investigate the effects of Nutlin-3 on cell growth and p53-Mdm2 interaction as well as to restore the tumour suppressive activity of p53 in NPC. The specific objectives of the study are: (1) To investigate the cytotoxicity effect of Nutlin-3 alone and in combination with cisplatin on C666-1 NPC and NPE cell growth; (2) To identify the potential mechanisms of cell death induced by cisplatin and/ or Nutlin-3 on C666-1 NPC cells; and (3) To investigate the effect of Nutlin-3 on the emergence of p53 mutations in C666-1 NPC cells.

2.5 Hypotheses of the Study

H_0: The reactivation of p53 in NPC cells does not suppress NPC cell proliferation, and in addition, Nutlin-3 combined with cisplatin does not further suppress the NPC cell proliferation effectively.

H_1: The reactivation of p53 in NPC cells suppresses NPC cell proliferation, and in addition, Nutlin-3 combined with cisplatin further suppresses the NPC cell proliferation more effectively.

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CHAPTER 3: MATERIALS & METHODS

3.1 Cell Lines and *in vitro* Culture

3.1.1 Cell Lines

Human non-tumorigenic NPE and NPC cell lines of different p53 status were used in this study. The NPC cell lines HK1 (EBV negative, mutant p53) (Huang *et al.*, 1980) and C666-1 (EBV positive, wt p53 with codon 72 polymorphism) were utilised. The immortalised NPE cell lines NP69SV40T (NP69) and NP460 hTERT (NP460) harbouring wt p53 with codon 72 polymorphism (Li *et al.*, 2006; Tsao *et al.*, 2002). The commercial available human colorectal carcinoma HCT116 cells (wt p53) (Brattain *et al.*, 1981) (ATCC CCL-247) and the metastatic breast adenocarcinoma MDA-MB-231 cells (ATCC HTB-26) were also used in the study.

3.1.2 Cell Culture Conditions

C666-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with 15% heat-inactivated foetal calf serum (FCS) (GIBCO). HK1 cells were cultured in 10% FCS RPMI medium. NP69 cells were cultured in keratinocyte-serum free medium (K-SFM) (GIBCO) supplemented with bovine pituitary extract and 0.16 ng/ml recombinant epidermal growth factor (GIBCO), while NP460 cells were maintained in a 1:1 ratio of defined K-SFM medium supplemented with growth factor (GIBCO) and EpiLife® Defined Growth Supplement (Cascade Biologics, Life Technologies, Carlsbad, CA, USA). HCT116 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) supplemented with 10% heat-inactivated FCS. All of the cell lines were maintained in exponential growth phase in the presence of 10 U of penicillin per ml (GIBCO) and 10 µg streptomycin per ml (GIBCO) at 37°C in 5% CO_2

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humidified atmosphere. All cell lines grow in an adherent manner except C666-1, which requires fibronectin coating to attach and adhere as monolayer. All cell lines were maintained in 10-cm culture plate (Techno Plastic Products (TPP), AG, Switzerland) and sub-cultured when they enter the logarithmic phase.

3.2 Determination of Optimal Cell Seeding Density

Prior to viability assay, a label-free cell-based assay was carried out using the xCELLigence system (ACEA Biosciences, Inc., San Diego, CA) to determine the optimal seeding density for each cell line. NP69 and NP460 cells were seeded at a density of $4 \times 10^3 - 3 \times 10^4$ cells/well, C666-1 at $5 \times 10^3 - 5 \times 10^4$ cells/well into the E-Plate 16 (ACEA Biosciences, Inc., San Diego, CA, USA). Cell titrates were prepared in duplicate, each containing 100 µl of complete culture medium per well. Duplicated wells containing media but no cells served as blank controls. Cells were monitored dynamically on an hourly interval for approximately six days (144 h) from the time of plating, at 37°C in 5% CO_2 atmosphere. Data was analysed using the Real-Time Cell Analyser (RTCA) software version 2.0 (ACEA Biosciences, Inc.). The seeding density was then applied for all subsequent cell viability assays carried out using 96-well plate. The optimal cell density used for seeding was prepared according to the equation 1;

$$C_1V_1 = C_2V_2 \text{ .....} \textit{Equation 1}$$

C_1 = original density of the cell suspension, before it gets diluted
C_2 = final density of the cell suspension, after dilution
V_1 = volume of cell suspension about to be diluted
V_2 = final volume of the cell suspension, after dilution

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3.3 Determination of Cell Viability by MTS Assay

The logarithmic growth phase cells were harvested and subjected to cell viability and density counting assays per the protocols described previously. Cell density of 1.5 x 10^4 NP69 and NP460, 3 x 10^4 C666-1 cells/ml were seeded in triplicate onto 96-well plates (TPP, AG, Switzerland). At 24 h post seeding, the used medium was aspirated and replenished with fresh medium containing various concentrations of cisplatin and/or Nutlin-3. Wells containing cells with 0.1% DMSO (Sigma-Aldrich St. Louis, MO, USA) or media but without cells served as vehicle control and blank, respectively. The final concentration of DMSO in each culture did not exceed 0.1%. Cells were subjected to treatment for 72 h at 37°C in 5% CO_2 atmosphere. The number of viable cells at the end of the treatment period was measured using CellTiter 96®Aqueous One Solution Cell Proliferation MTS Solution (Promega, Madison, WI, USA). The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium] is bioreduced by mitochondrial reductase and combined with enhancer phenazine ethosulfate to produce a stable purple coloured water-soluble formazan. The colour intensity of the formazan is directly proportional to the number of viable cells present in the culture. The reaction was allowed to occur within 4 h at 37°C in dark. By using the EnVision multilevel plate reader (PerkinElmer, Waltham, MA, USA), the absorbance was read at 490 nm followed by subtraction with non-specific absorbance at 630 nm. Cell viability was expressed as percentage relative to untreated or vehicle control with arbitrarily assigned 100% viable ([Average OD treated - Average OD blank] x 100% / Average OD untreated or vehicle control). The IC_{50} value was graphically obtained from the dose-response curves, analysed by Microsoft Excel and/or GraphPad Prism Version 5 (GraphPad Software Inc., San Diego, CA, USA). The experiments were repeated three times to establish nine data points.

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3.4 Determination of Anchorage-independent Growth of NPC Cells by Soft Agar Colony Formation Assay

Soft agar colony formation assay was used to measure C666-1 cells' efficiency of anchorage-independent colony formation in soft agar. A two-layer soft agar (DNA grade Seakem Agarose Powder, Lonza, Rockland, USA) culture system with plating of cisplatin and/or Nutlin-3 treated C666-1 cells in six-well plate (TPP, AG, Swistzerland) was used in this study. Cell seeding was performed as per the MTS cell viability assay described previously. In a 96-well plate (TPP, AG, Swistzerland), C666-1 cells were seeded at density 5 x 10^4 cells/ml in 100 µl per well and allowed to grow overnight. At 24 h post seeding, the old medium was aspirated and replenished with fresh medium containing cisplatin and/or Nutlin-3. The sensitivity of the assay to DMSO was also investigated. Wells containing cells with 0.1% DMSO or media but without cell served as vehicle control and blank, respectively. At 24 h post treatment, cells together with treatment medium were plated into the soft agar in 6-well plates, comprising of a layer of 0.3% agar in complete media and 0.6% agar as base layer. Cells were subjected to treatment at 37°C in 5% CO_2 atmosphere. The cultures were monitored microscopically for colonised formation for 7–21 days. Cultures were refreshed with 0.2 ml of complete medium twice a week. Anchorage-independent growth was measured by counting the score of viable colonies using an Olympus stereo microscope model SZX7 (Olympus, Tokyo, Japan), equipped with Image Pro Plus AMS version 6.3 (Media Cybernetics, Inc. Rockville, MD, USA). The computerised-imaging analysis accurately measured the area of each colony at 60 µm in diameter or larger to size the changes in colony forming and growth observed at the time of drug application. Colonies with a minimum diameter of 60 µm, area 2800 µm² and roundness score ranging from 0.25 to 0.50 (roundness = $4A / \pi D2$; A = area; D = maximum diameter; with 1.0 indicating a perfect circle) were counted in order to exclude abortive colonies (Jones *et al.*, 1985).

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3.5 Evaluation the Effects of Nutlin-3 on p53 Pathway by Western Blotting

3.5.1 Cell Preparation and Treatments

Cell lines with different p53 status (wt p53: C666-1 and HCT116; mt p53: HK1 and MDA-MB-231) were used to determine the effects of Nutlin-3 on the activation of p53 pathway. The effects of Nutlin-3 on p53 pathway were evaluated on the expression of proteins p53, p21, Mdm2, BAX and Bcl2. One million cells were sub-cultured onto a 10 cm-culture plate (TPP, AG, Switzerland). At 24 h post plating, the old medium was removed and the cultures were allowed to expand in medium containing 10 μM Nutlin-3 or left treated with 0.1% DMSO at 37°C in a 5% CO_2 atmosphere. Additionally, the effects of Nutlin-3 on the activation of p53-mediated apoptotic genes were performed on C666-1 cells. Proteins expression was detected using monoclonal antibodies anti-p53, anti-p21 (Waf1/Cip1), anti-Mdm2, anti-BAX, anti-PUMA and anti-PARP. β-actin served as loading control. One million cells were sub-cultured onto a 10 cm-culture plate. At 24 h post plating, the used medium was removed and the cultures were allowed to expand in medium containing 33.33 μM cisplatin and/or 10 μM Nutlin-3 at 37°C in 5% CO_2 atmosphere. Cultures containing basal media or 0.1 % DMSO served as untreated and vehicle control, respectively. At 24 h post treatment, the floating and adherent cells were harvested and subjected for protein extraction.

3.5.2 Cell Lysis and Protein Extraction

At 24 h post treatment, the culture medium and floating cells were harvested. Adherent cells were suspended in 1.0 ml of 0.25% trypsin-EDTA. Cells were washed twice with 5 ml of pre-cooled 1x PBS (without Ca^{2+}, Mg^{2+}) and concentrated by centrifugation at 1000 rpm for 5 min. Cells were lysed with 70 μl of pre-cooled RIPA lysis buffer containing 1x RIPA buffer, 2 mM DTT, 0.2 mM PMSF (Bio-Rad Laboratory, USA) and protease inhibitor cocktail (Roche Diagnostics GmbH,

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Mannheim, Germany). Cells were agitated for 10 min using a vortex, followed by rotating at 100 rpm at 4°C for 40 min. To remove cellular debris and unwanted DNA, the lysates were centrifuged at 13 000 rpm at 4°C for 30 min. The protein-containing supernatant was collected for protein quantitation.

3.5.3 Protein Quantitation

Protein concentration was determined by colorimetric Bradford method using Bio-Rad protein assay kit (Bio-Rad Laboratory, USA), which contains dye, phosphoric acid and methanol. The extracted protein and blank (distilled water) were diluted in a ratio of 1:400 with dH_20 and Bio-Rad dye reagent. The samples were well-mixed and incubated in dark at room temperature for 5 min. The absorbance at OD_{595nm} was measured versus reagent blank. The dye-binding assay involves the binding of acidic Coomasie brilliant blue G-250 dye to the aromatic amino acid residues of solubilised protein. Protein concentration measurements were based on the degree of colour changes in response to the formation of a measurable acidic solution of Coomasie brilliant blue G-250 dye shifts from 465 nm to 595 nm when the binding occurred.

3.5.4 SDS-PAGE Gel Electrophoresis

Cell lysates were electrophoresed using polyacrylamide gels consisting of 5% w/v stacking gel (top-layer) and 10% w/v resolving gel (lower-layer) for targeting proteins with molecular weight between 10–90 kDa. The gels were prepared in a mixture of dH_2O, acrylamide, Tris-Cl, SDS, APS and TEMED (Table 3.1) (Bio-Rad, Hercules, CA, USA). The gel solution was allowed to polymerise at room temperature (RT) for 30 min, with a comb fitted into the stacking gel layer to create wells for sample loading. A volume (µl) of 10 µg protein lysate was diluted in 1:3 ratio volume of loading dye,

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which consisted of 1x SDS and 4 x 2.0% mercaptoethanol. The mixture was heated at 95°C for 5 min to further denature the proteins and enhance binding to SDS. The protein lysate (10 µg) was loaded into the inner lanes in parallel with a volume of 5 µl Full-range Rainbow™ Molecular Weight Marker (Amersham, GE Healthcare UK), ranging from 12–225 kDa, was loaded into the outer lane of each gel. The gel was immersed in 1x running buffer (Tris-glycine, 10% SDS, pH 8.3) and ran at 100V for about 90 min or until the marker reached the bottom of the gel.

Table 3.1: Tris-glycine SDS-polyacrylamide resolving and stacking gels

Mixture of reagent	10% resolving gel	5% stacking gel
	Volume (ml)	Volume (ml)
dH_2O	4.000	2.100
30% acrylamide (29:1)	3.300	0.500
Tris-Cl 0.5M, pH 8.8	2.500	-
Tris-Cl 0.5M, pH 6.8	-	0.380
10% SDS	0.100	0.030
10% APS	0.100	0.030
TEMED	0.004	0.003
Total	**10.004**	**3.043**

3.5.5 Immunotransfer and Immunoblotting

To prepare the protein for antibody detection, the size-separated proteins were electro-transferred onto a polyvinylidene fluoride (PVDF) nitrocellulose membrane (Millipore, Bedford, MA) using fresh-prepared cold transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% w/v SDS and 20% v/v methanol), ran at 15V at RT for overnight. Following protein transfer, the membrane was subjected for blocking in order to prevent non-specific interactions between the membrane and the antibodies. Blocking of non-specific antibody binding was done using 5% bovine serum albumin (BSA) (Sigma-Aldrich) or non-fat dry milk (NFM) (Sunlac, Malaysia) in Tris-buffered saline (TBS) (5M NaCl, 1M Tris, pH 7.3) containing 0.1% Tween 20 (TBST) (Sigma-Aldrich), at RT for 1 h. Blocking buffer was washed away with 10 ml of 1x TBST, 5

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min per wash. The membrane was probed with primary antibodies (specific for the targeted proteins) in 1x TBST containing 5% BSA or NFM. The reaction occurred at RT for 1 h. Monoclonal antibodies used were p53 (Promega Corporation, Madison, Wisconsin, USA, 1:5000), p21 (Waf1/Cip1), BAX, Bcl2, (Cell Signalling Technology, Inc., MA, USA, 1:5000), Mdm2 (Santa Cruz Biotechnology, Inc., CA, USA, 1:5000), PUMA and PARP (Cell Signalling, 1:2000). β-actin (Santa Cruz, 1:5000) was used as loading control. The unbound antibodies were removed by washing as described previously. Secondary antibody reactions (specific for targeted protein) were performed with anti-mouse (Promega, 1:2000) or anti-rabbit (Promega, 1:15000) horseradish peroxidase conjugated immunoglobulin (IgG), at RT for 1 h. Excess secondary antibodies were removed by washing as described previously. The enzyme-linked secondary antibody, which is specific to the primary antibody, is visually detectable when exposed to chemiluminescent agent under chemiluminescent imaging.

3.5.6 Quantitation of Band Densities

Chemiluminescent imaging was employed for visualization of targeted immunoblotted proteins. Chemiluminescent reaction was performed using Western Lightning® Plus Enhanced chemiluminescence (ECL) substrate (PerkinElmer, Waltham, MA, USA), followed by detection using ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Buckinghamshire, UK). The Full-range Rainbow™ Molecular Weight Marker, a mixture of individually coloured proteins of defined size, was used to determine the size of the proteins (kDa). Capture-visualised signals appeared as dark bands proportional to the amount of protein, relative and normalised with respect to the β-actin product, which was probed to monitor protein loading and transferring. Densitometric analysis was performed on Alpha Imager HP System with the AlphaView software (Alpha Innotech Corporation, San Leandro, CA, USA).

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3.6 Polymerase Chain Reaction (PCR) and DNA Sequencing

The reliable molecular methods used for rapid gene mutation detection involve primers design, DNA extraction, PCR and gene sequencing. PCR (Whitney *et al.*, 2004) was performed according to the three major profiles: denaturation, primer annealing and extension (Mullis *et al.*, 1986). DNA denaturation is the first step in every PCR reaction. The samples were heated to 94°C in two stages, 5 min and 30 sec, to separate double-stranded DNA (dsDNA). The optimal annealing temperature, T_a = [0.3 x (T_m of primer) + 0.7 x (T_m of product) - 25] was set about 5°C below the lowest of the primer pairs' melting temperature, T_m = 2(A + T) + 4(G + C) (Rychlik *et al.*, 1990). In annealing the primers to single-stranded DNA, hydrogen bond bridges the complementary base-pair nucleotides. In the beginning of extension, the temperature was raised to 72°C for two stages, 30 sec and 5 min. Taq polymerase was needed for nucleotides binding at the 3' end of each primer to catalyse the synthesis of new double-stranded DNA. Prior to next cycle, the temperature was reset at 94°C at the end of each cycle. The cycles were repeated 35 times to generate 68 billion copies of targeted dsDNA.

3.6.1 Primer Design and Synthesis

The success of PCR is T_a dependent, at which the dsDNA dissociates to become single stranded. Precise prediction of T_a can be achieved by calculating the T_m of the primers. T_m is definable by computing the percentage of GC (Guanine–Cytosine) content of the primers. Primer design is important in improving PCR product yield. In this study, primers targeting the 2^{nd} to 11^{th} exons of p53 gene (Table 3.2) were tailored using Primer Premier 6.12 software (Premier Biosoft International) (Innis and Gelfand, 1990).

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Table 3.2: Oligonucleotide primer sequences priming the 2nd to 11th exons of p53 gene

Exon	Forward primers (5' → 3')	Reverse primers (5' → 3')	Product (bp)
2	AGC TGT CTC AGA CAC TGG CA	GAG CAG AAA GTC AGT CCC ATG	317
3+4	AGA CCT ATG GAA ACT GTG AGT GGA	GAA GCC TAA GGG TGA AGA GGA	631
5	CGG AAT TCT TAT CTG TTC ACT TGT GCC C	CGG GAT CCA CCC TGG GCA ACC AGC CCT G	284
6	CGG AAT TCG GTC CCC AGG CCT CTG ATT CCT	CGG GAT CCA CCC GGA GGG CCA CTG ACA AC	215
7	CTG CTT GCC ACA GGT CTC	TGG ATG GGT AGT AGT ATG GAA G	283
8	CGG AAT TCT TGG GAG TAG ATG GAG CCT	CGG GAT CCC TCC TCC ACC GCT TCT TGT CCT	259
9	AGC AAG CAG GAC AAG AAG CG	CCA GGA GCC ATT GTC TTT GA	304
10	CTC AGG TAC TGT GTA TAT ACT TAC	ATA CTA CGT GGA GGC AAG AAT	351
11	TCC CGT TGT CCC AGC GTT	TAA CCC TTA ACT GCA AGA ACAT	476

The sequences were blasted and the recruiting important criteria re-evaluated, include GC content, base-pair length, secondary structures, number of repeat bases and primers' T_m (Chavali *et al.*, 2005). Since GC pairing is more stable than AT pairing, primer sequences were fitted into 40–60% GC base composition. To prevent "ends breathing" and promote priming efficiency, the 3' end was tailored ending with C or G, CG or GC. To avoid mispriming and non-specific binding of G's and C's at the 3' end, primer length was tailored at 17–28 base pairs and fitted at T_m 55–80 °C. The primers were tailored to bracket the initiation and the end region for amplification.

3.6.2 DNA Extraction

Cells were maintained to reach 80% confluence at logarithmic growth phase. The adherent cells were suspended with 1.0 ml of 0.25% trypsin-EDTA, washed twice with 5.0 ml pre-cooled phosphate buffered saline (PBS) and concentrated by centrifugation at 1000 rpm for 5 min. Cell viability and density were determined using 0.4% tryphan blue stain. Genomic DNA was extracted using DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations. Cell lysis was performed in 300 µl Cell Lysis Solution containing 10 µl of 20 mg/ml proteinase K; reaction was allowed to

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occur at 56°C for 1 h. The lysate was subjected to protein precipitation in 100 µl Protein Precipitation Solution. After centrifugation at 13 000 rpm for 1 min, the supernatant containing the unwanted protein was discarded, 300 µl of 100% Isopropanol (2-propanol) were added for DNA precipitation. After washing with 300 µl of 70% ethanol, pure DNA was hydrated rapidly in 50 µl DNA Hydration Solution at 65°C for 5 min.

3.6.3 DNA Quantitation

The concentration of extracted DNA was quantified using Nanodrop™ 8000 spectrophotometer V2.1.0 (Thermo Fisher Scientific, Waltham, MA, USA). The final concentration was calculated from the average of triplicate readings. The DNA was then diluted to 100 ng/µl in a total volume of 50 µl with nuclease free H_2O.

3.6.4 DNA Amplification

The coding regions of the 2^{nd} to 11^{th} exons of the p53 gene were amplified exon-by-exon using the Bio-Rad DNA thermal cycler (Hercules, CA, USA). Parameters that needed to be optimised were the DNA concentration, T_a and total number of cycles. PCR reactions were set in a sterile 0.5 µl-flat cap Eppendorf PCR tube, containing 10 µl of 2x Dynazyme (BioLabs, New England), 1.0 µl of 10 µM of each forward and reverse primers, and 2.0 µl of 100 ng/µl DNA. The final reaction volume was 20 µl (Table 3.3). The PCR reactions were repeated for 35 cycles, consisted of enzyme activation and template denaturation at 94°C for 5 min followed by 30 sec, gradient annealing ranging from 50–65°C for 30 sec, and two extension phases set at 72°C for 30 sec and 72°C for 5 min (Table 3.4).

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Table 3.3: PCR master mix used for DNA amplification

PCR master mix	One reaction (µl)
2x Dynazyme	10.0
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
Nuclease free H_2O	6.0
Template DNA (100 ng/µl)	2.0
Final volume/ reaction	20.0

Table 3.4: Thermal cycling profile

	Temperature °C / time
Initial denature	94°C / 5 min
Denaturation	94°C / 30 sec
Annealing (T_a)	50–65°C / 30 sec
Extension	72°C / 30 sec
Final extension	72°C / 5 min
Ending	4°C / overnight

3.6.5 PCR Products Evaluation

The quality and purity of the resulting PCR products were evaluated on an ethidium bromide-stained 1.5% agarose gel. Agarose gel was prepared by boiling 0.75 g D1-low EEO agarose powder (Lab. Conda, Pronadisa Spain) in 50 ml of 1x Tris-Borate Buffer (TBE) (Sigma-Aldrich). Agarose gel containing 1.0 µl ethidium bromide (Sigma-Aldrich) was casted onto gel tray assembled with comb and allowed to solidify at RT. Mixture of PCR product with 6x loading dye (Bromophenol blue, SolGent™, Korea) in a 2:1 ratio was loaded for electrophoresis. A mixture of 8.0 µl of 100 bp ladders (Life Technologies, Grand Island, NY, USA) to 2.0 µl of 6x loading dye was loaded. The products were electrophoresed at 120V for 20–30 min in 1x TBE buffer. Band visualisation was detected under ultraviolet (UV) illumination using AlphaImager Enhanced Gel-Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA, USA).

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3.6.6 DNA Purification

Prior to Sanger sequencing, PCR products were further purified to remove surplus materials such as excessive PCR master mixed reagents and primer dimers using Qiagen QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA). Purification was performed using 5x volume of sodium acetate-containing Buffer PB (guanidine hydrochloride and isopropanol). Excessive waste was discarded by centrifugation at 13 000 rpm for 1 min. Membrane bound DNA was washed with 750 µl ethanol-containing Buffer PE. Excessive ethanol was discarded by centrifugation at 13 000 rpm for 1 min. Pure DNA was eluted in 50 µl pre-warmed dH_2O and collected by centrifugation at 13 000 rpm for 1 min.

3.6.7 DNA Concentration and Purity

The DNA mass was estimated using Low DNA Mass Ladder (LDML) (Life technology, Invitrogen) on an ethidium bromide-stained 2.0% agarose gel; and re-quantitated using Nanodrop 8000 spectrophotometer V2.1.0. The ethidium bromide-stained 2.0% agarose gel was prepared as drescribed in section 3.6.5. Electrophoresing a volume of 2 µl LDML resulted in six bands bearing a total of 470 ng DNA ranging from 10–200 ng (Figure 3.1). A mixture of DNA product and LDML to 6x loading dye was prepared in 1:1 and 2:1 ratio, respectively. The products were electrophoresed at 120V for 20–30 min in 1x TBE buffer. The DNA mass was estimated relative to the LDML under UV illumination using AlphaImager Enhanced Gel-Documentation System. The DNA was then diluted with nuclease free H_2O to 20 ng/µl in a total volume of 10 µl.

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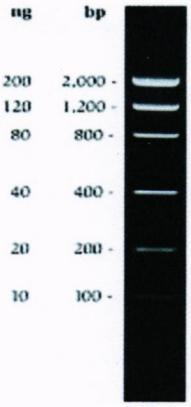

Amount of DNA (ng) in each band

	Volume of Low DNA Mass Ladder		
Fragment size	2 µl	4 µl	8 µl
2000 bp	100 ng	200 ng	400 ng
1200 bp	60 ng	120 ng	240 ng
800 bp	40 ng	80 ng	160 ng
400 bp	20 ng	40 ng	80 ng
200 bp	10 ng	20 ng	40 ng
100 bp	5 ng	10 ng	20 ng

Quality Control:
Agarose gel analysis shows that all bands (2000, 1200, 800, 400, 200, 100 bp) are clearly distinguishable. Sharpness and relative intensity of the bands and the absence of background are evaluated relative to a control lot by agarose gel analysis.

Figure 3.1: Low DNA Mass Ladder: 4 µl/ application in 2.0% agarose gel stained with ethidium bromide. Adapted from Low DNA Mass Ladder, Invitrogen by Life Technologies *https://tools.thermofisher.com/content/sfs/manuals/Low_DNA_Mass_Ladder_man.pdf*

3.6.8 DNA Sequencing

The p53 status of the cell lines were determined by genomic DNA sequencing. Pure DNA products were outsourced to First Base Sdn. Bhd. (Malaysia) for sequencing. The electropherogram of each exon was blasted with Basic Local Alignment Search Tool (BLAST), aligned against human p53 sequences (X54156) published in NCBI GenBank. The nucleotide variants were analysed using Mutation Surveyor V4.0.7 (SoftGenetics, LLC., PA, USA).

3.7 p53 Knockdown with Small-hairpin RNA

3.7.1 Small-hairpin RNA Lentiviral System

Integration of small-hairpin RNA (shRNA) into C666-1 cells for stable p53 gene silencing was performed using Lentivector-based shRNA system (Mission® Sigma-Aldrich, St Louis, MO, USA). The shRNA construct consisting of pLKO.1-puro vector derived from human immunodeficiency virus HIV-1 was created by transferring a U6 promoter-shRNA cassette into a lentiviral backbone with puromycin as the selective marker (Figures 3.2 and 3.3). The p53 target sequences of each shRNA lentiviral particle are listed in Figure 3.4 and Table 3.5.

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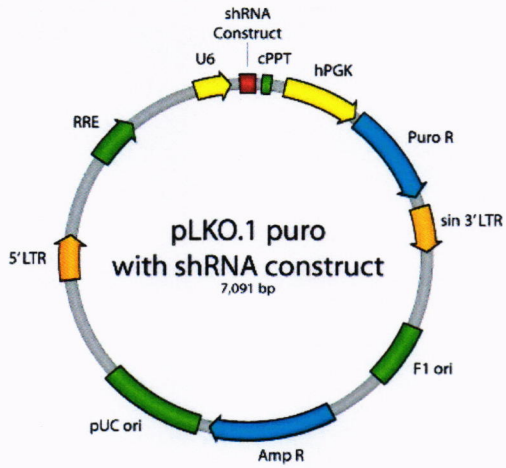

Description	Vector Elements
U6	Human U6 promoter drives RNA Polymerase III transcription for generation of shRNA transcripts
cPPT	Central polypurine tract, cPPT, improves transduction efficiency by facilitating nuclear import of the vector's pre-integration complex in the transduced cells
hPGK	Human phosphoglycerate kinase promoter drives expression of puromycin
Puro R	Puromycin resistance gene for selection of pLKO.1 plasmid in mammalian cells
sin 3'LTR	3' Self-inactivating long terminal repeat
f1 ori	f1 bacterial origin of replication
Amp R	Ampicillin resistance gene for selection of pLKO.1 plasmid in bacterial cells
pUC ori	pUC bacterial origin of replication
5'LTR	5' long terminal repeat
RRE	Rev response element

Figure 3.2: Vector Map pLKO.1-puro containing a shRNA insert and description. Adapted from Addgene Plasmid 10878. Protocol Version 1.0. *http://www.addgene.org/tools/protocols/plko*

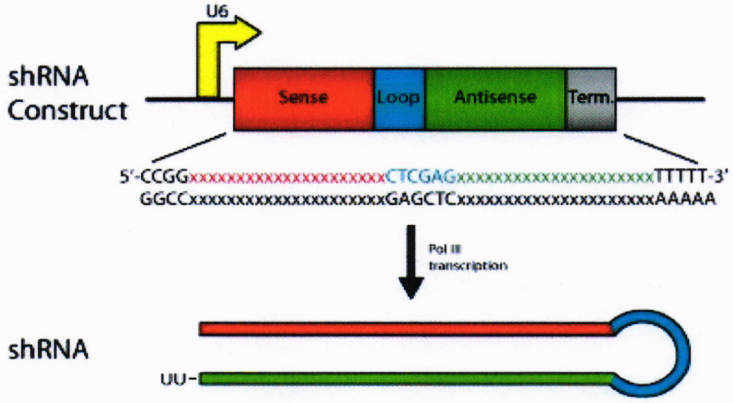

Figure 3.3: Design of p53 shRNA constructs. The shRNA contains 21 "sense" bases that are identical to the p53 gene, a loop containing an Xhol restriction site, and 21 "antisense" bases that are complementary to the 21 "sense" bases. Adapted from Addgene Plasmid 10878. Protocol Version 1.0. *http://www.addgene.org/ tools/protocols/ plko*

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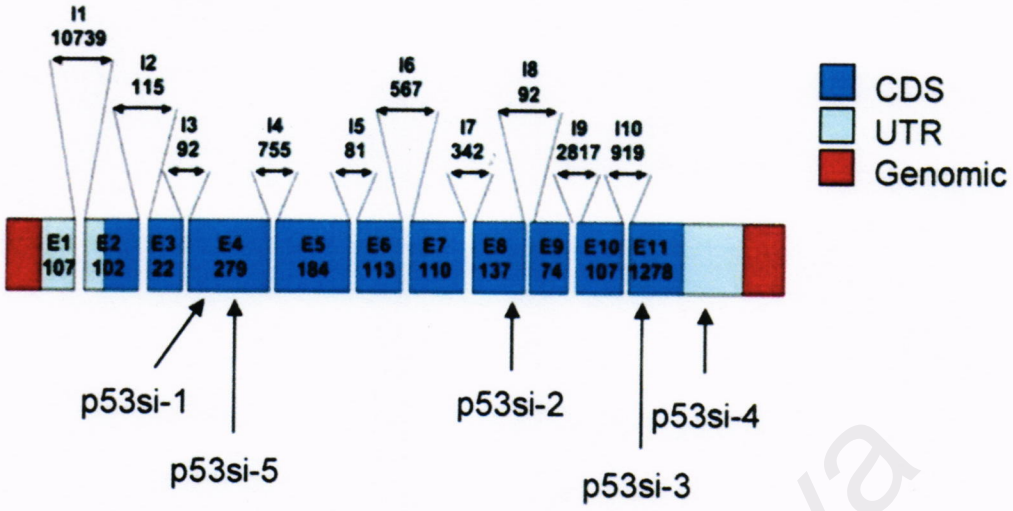

Figure 3.4: The p53 shRNAs target different sequence of the p53 gene. All the shRNAs target the coding sequence of the p53 gene with exception of p53si-4, which targets the 3′ UTR. Adapted from Addgene Plasmid 10878. Protocol Version 1.0. *http://www.addgene.org/tools/protocols/plko*

Table 3.5: p53 shRNA target sequences of p53 gene

Vector	p53 shRNA	Label	Target region	Targeted Sequences
pLKO.1-puro	p53si-2	D3	CDS	5′-CACCATCCACTACAACTACAT-3′
	p53si-3	C12	CDS	5′-CGGCGCACAG AGGAAGAGAAT-3′
	p53si-4	E1	3′ UTR	5′-GAGGGATGTTTGGGAGATGTA-3′
	p53si-5	E2	CDS	5′-GTCCAGATGAAGCTCCCAGAA-3′
	Non-specific	NS	-	5′-CAACAAGATGAAGAGCACCAA-3′

A, adenine; C, cytosine; CDS, coding DNA sequence; G, guanine; T, thymine; UTR, untranslated region

Table 3.6: Lentiviral transduction mixture used for co-transfecting HEK-293T cells

shRNA plasmid vector	Packaging plasmids		Lipofectamine 2000	Optimum	DME media supplemented with 10% FCS
	psPAX2	pMD2.G			
5 µg	5 µg	5 µg	25 µl	500 µl	6 ml

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3.7.2 Generation of Lentiviral Transduction Particle

Lentiviral stocks were generated by co-transfecting the HEK-293T cells (ATCC® CRL-3216™) (American Type Culture Collection, Manassas, VA, USA) with plasmid vector, the psPAX2 packaging plasmids (Addgene plasmid 12260) and pMD2G envelope plasmid (Addgene plasmid 12259) (Addgene, Inc., Cambridge, MA, USA) using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. HEK-293T cells were plated at a density of 1.5 x 10^6 onto a 10 cm-culture plate. At 24 h post plating, old medium was removed; freshly prepared transduction particle (Table 3.6) was added drop by drop directly into the HEK-293T culture. Culture was allowed to expand in transduction particle-containing medium for 48 h at 37°C in 5% CO_2 atmosphere. At 48 h post transfection, virus-containing medium was collected and purified using 0.45 μm filter.

3.7.3 p53 Gene Knockdown and Validation

C666-1 cells were plated at a density of 1.5 x 10^6 into a 10 cm-culture plate. At 24 h post plating, used medium was removed; culture was allowed to expand in medium containing equal volume of viral particles and cells (1:1 ml) supplemented with 8 μg/ml polybrene (Sigma-Aldrich), for 15 h at 37°C in 5% CO_2 atmosphere. p53 knockdown was performed using four lentiviral-based shRNA constructs p53si-E1, -E2, -C12 or -D3, inclusive empty vector pLKO.1 (Vector), pLKO.1-GFP (GFP), Non-specific pLKO.1 (NS) and parental C666-1 cells with and without polybrene. At 15 h post transfection, the used medium was aspirated and replenished with fresh medium. Establishment of p53 knockdown was monitored by measuring the green fluorescent protein (GFP) signal. The efficacy and effects of knockdown on the expression of p53 and its transcriptional target proteins p21 and Mdm2 was verified by Western blotting followed by densitometry analysis. Prior to Western blotting, the lenti-shΔp53 C666-1

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cells were allowed to expand in basal medium for at least 72 h. One million lenti-shΔp53-transduced C666-1 cells were plated onto a 10 cm-culture plate. At 24 h post plating, old medium was removed and the cultures were allowed to expand in medium containing 10 μM Nutlin-3 or left treated with 0.1% DMSO at 37°C in 5% CO_2 atmosphere. At 24 h post treatment, the floating and adherent cells were harvested and subjected for protein extraction per the protocols described previously. β-actin served as loading control.

3.8 Investigating Mechanisms of Cell Death by High Content Analysis of Apoptosis

Programmed cell death, apoptosis, is a natural way of eliminating unhealthy cells in multicellular organisms. The phospholipid component of phosphatidylserine (PS) is attached on the inner-leaflet of viable cell membranes. Soon after apoptosis, the loss of plasma membrane integrity results in translocation of PS exposed to membrane surface. Annexin V is a calcium-dependent phospholipid-binding protein, with a strong and specific affinity to PS. The externalisation of PS on apoptotic cells is detectable by FITC-labelled Annexin V stain using fluorescence microscopy. The logarithmic growth phase C666-1 cells were seeded at a density of 3 x 10^4 cells/ml onto ViewPlate-96 Black 96-well plates (PerkinElmer, Waltham, MA, USA). At 24 h post seeding, the old medium was aspirated and replenished with fresh medium containing 33.33 μM cisplatin and/or 10 μM Nutlin-3. Cells cultured with 0.1% DMSO or basal media served as vehicle control for Nutlin-3 and cisplatin treatment. Cells were subjected to treatment for 48–72 h at 37°C in 5% CO_2 atmosphere. At the end of treatment, cells were stained with Annexin V-FITC (apoptosis), propidium iodide (viability) and Hoechst 33342 (counter stain) (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Well-to-well imaging with three filter channels (DAPI, FITC and TRITC) was

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performed using Metamorph screening acquisition module, on a Nikon Ti-ECLIPSE inverted fluorescent microscope (Nikon Corporation, Tokyo, Japan), at 200x magnification. Nine fields were imaged and scored for each well using Metamorph software version 7.7.0.0 (Molecular Devices, Downingtown, PA, USA). The percentage of apoptotic cells were calculated from triplicate wells.

3.9 Investigation of Drug Resistance Emergence in Response to Nutlin-3 Treatment

3.9.1 Establishment of Nutlin-3-resistant NPC Cells

Nutlin-3-resistant cells were generated by propagating parental C666-1 cells in stepwise ascending concentrations (10, 20 and 40 μM) of Nutlin-3 for a number of passages (7–36) over a period of up to six months (Figure 3.5). Concentration at 10 μM Nutlin-3, IC_{50} parental C666-1 (N/0), was selected as a start. Cells were allowed propagate in medium containing Nutlin-3, sub-cultured at exponential growth phase at low density. Prior to MTS viability assay and gene sequencing, Nutlin-3-treated cells were cultivated in basal medium for at least 72 h. Cells were seeded at a density 3 x 10^4 cells/ml and 100 μl were added to each well of 96-well plates. At 24 h post seeding, the used medium was aspirated and replenished with fresh medium containing various concentrations of Nutlin-3. Cells were allowed to be treated for 72 h at 37°C in 5% CO_2 atmosphere. Wells containing cells with 0.1% DMSO or media but without cell served as vehicle control and blank, respectively. Cell viability of Nutlin-3-resistant sublines relative to control parental C666-1 cells was determined by MTS viability assay following the protocol described in section 3.3. The resistance index (RI, IC_{50} resistant cell / IC_{50} sensitive cell) was calculated to determine the degree of acquired resistance to its relative control parental C666-1 cells.

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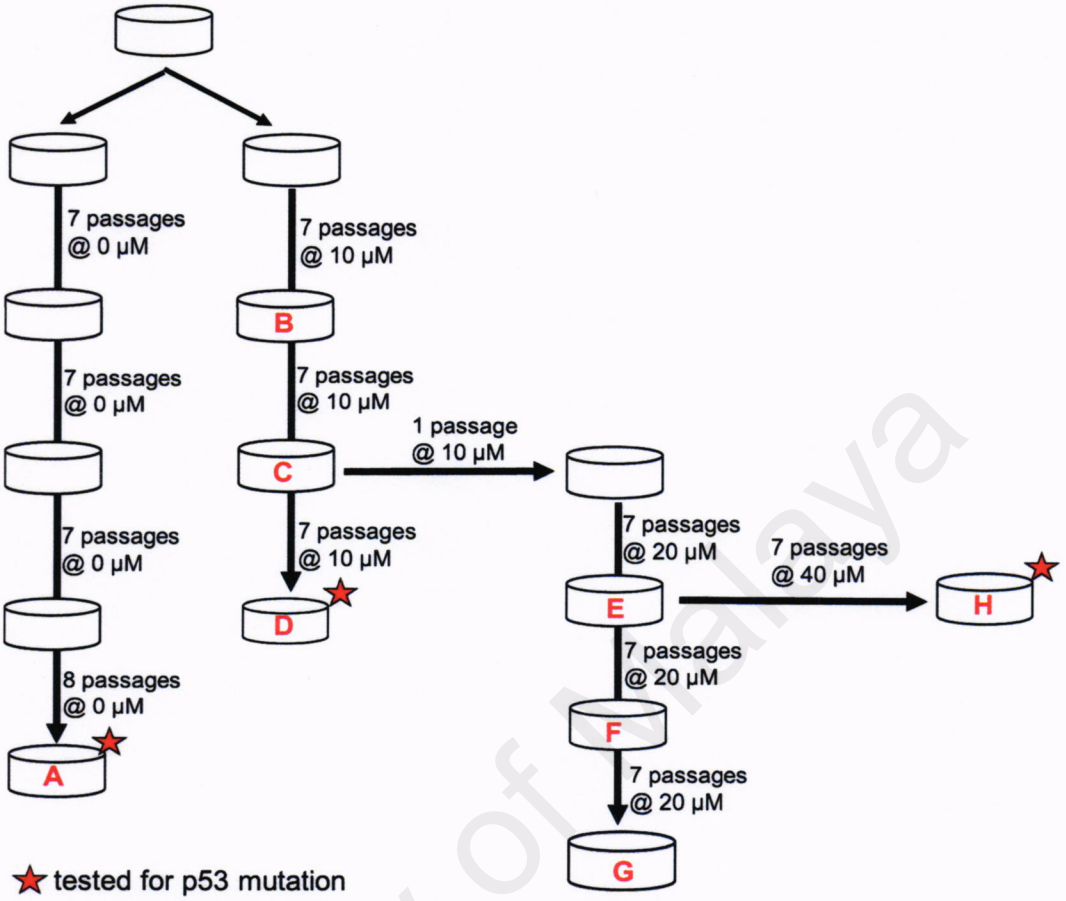

Figure 3.5: The diagram of experimental design to establish Nutlin-3-adapted NPC cell sublines. Nutlin-3-adapted C666-1 cell sublines were established by treatment of C666-1 cells with 10 to 40 µM Nutlin-3 over a period of up to six months. The sublines tested for p53 mutations are labelled (★).

3.9.2 Determination of p53 Mutation in Nutlin-3-resistant Cells

The p53 status spanning from exons 2nd to 11th of the Nutlin-3-resistant C666-1 cells was determined by genomic DNA sequencing. PCR and DNA sequencing was performed as described in section 3.6. The electropherogram of each exon was blasted with Basic Local Alignment Search Tool (BLAST), aligned against human p53 sequences published in GenBank (X54156). The nucleotide variants were analysed using Mutation Surveyor V4.0.7 (SoftGenetics, LLC., PA, USA).

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3.10 Statistical Analysis

Data was analysed by Microsoft Excel and/or GraphPad Prism Version 5 (GraphPad Software Inc., San Diego, CA, USA). Data are shown as means ± standard deviation (SD) of at least three independent experiments, unless otherwise specified. Statistical significance was measured using Student's paired t-test and (*) p values<0.05, (**) p values<0.005 were considered to be statistically significant.

3.11 Summary of Study Design

There were different cell lines and several methods used in the study. The cell lines, various techniques and how the methodology are linked together in the overall study were summarised in a flowchart (Figure 3.6).

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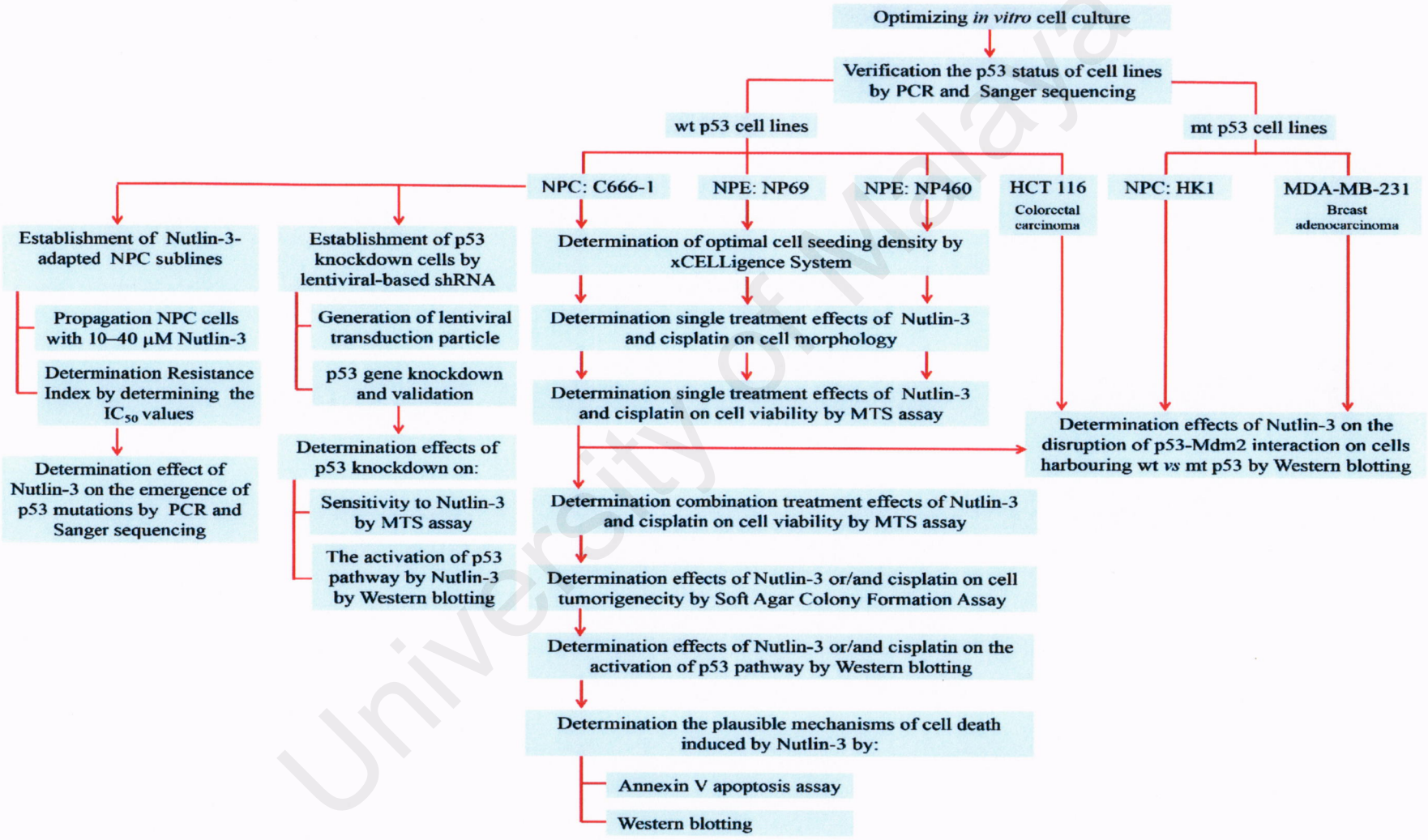

Figure 3.6: Summary of the overall study design

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CHAPTER 4: RESULTS

4.1 Status of p53 Mutation in NPC and NPE Cells

In the present study, p53 sequences in NPC (C666-1; HK1) and NPE (NP69; NP460) cell lines were first investigated by sequencing exons 2 through 11 of the p53 gene. The p53 sequences in C666-1 cells had a single G>C base substitution detected at position 12139 at codon 72 of the 4^{th} exon, while no alteration was detected in other exons (Table 4.1). An identical alteration was detected in the NP69 and NP460 cell lines. However, a homozygous C>G base substitution at codon 130 of exon 5 was detected in HK1 cells. Overall, these results suggest that p53 mutation was only detected in HK1 cells, while C666-1, NP69 and NP460 cells retained the wt p53 status whilst expressing a polymorphism at codon 72, common in wt p53 gene.

4.2 Cytotoxicity Effect of Cisplatin and Nutlin-3 on NPC and NPE Cells

The optimal cell seeding densities for NP69 and NP460 were determined to be at 1.5 x 10^4 cells/well and 3 x 10^4 cells/well for C666-1 (Figure 4.1) where the exponential phase during the treatment period became evident at the said densities. The selected seeding densities were applied for all subsequent MTS viability assays.

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Table 4.1: Summary of p53 mutation status of exons 2nd through 11th of NPC and NPE cell lines

Exons p53	CDS	NP69	NP460	C666-1	HK1
2	11717..11790	Nm	Nm	Nm	Nm
3 + 4	11906..11927 12021..12299	†	†	†	Nm
5	13055..13238	Nm	Nm	Nm	††
6	13320..13432	Nm	Nm	Nm	Nm
7	14000..14109	Nm	Nm	Nm	Nm
8	14452..14588	Nm	Nm	Nm	Nm
9	14681..14754	Nm	Nm	Nm	Nm
10	17572..17678	Nm	Nm	Nm	Nm
11	18599..18680	Nm	Nm	Nm	Nm

†, a base change of C**G**C to C**C**C in 4th exon at 12139 bp with no mutation detected; ††, a homozygous point mutation of **C**TC to **G**TC in 5th exon at codon 130; CDS, coding DNA sequences; Nm, No mutation detected.

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Figure 4.1: The real-time kinetic growth and changes in Cell Index acquired from xCELLigence system. The Cell Index over proliferation time of cell lines (**A**) NP69 (**B**) NP460 and (**C**) C666-1. The graphs shown are representative of duplicate experiments. The mean Cell Index (SD bars) relative to no cell control are shown in an hourly interval for approximately 144 h from the time of plating.

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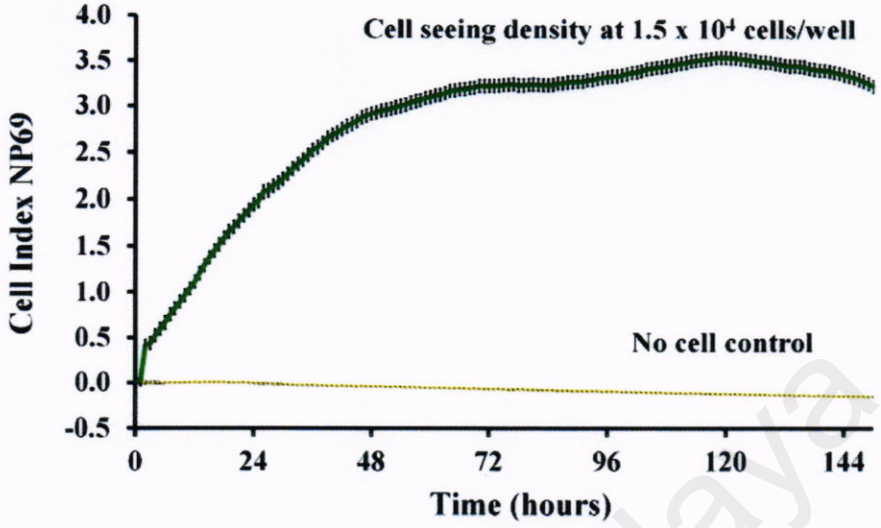
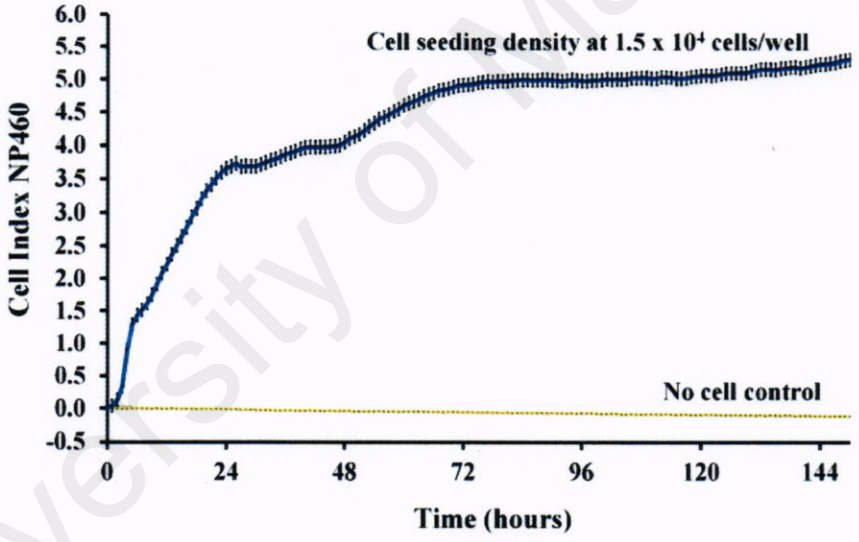
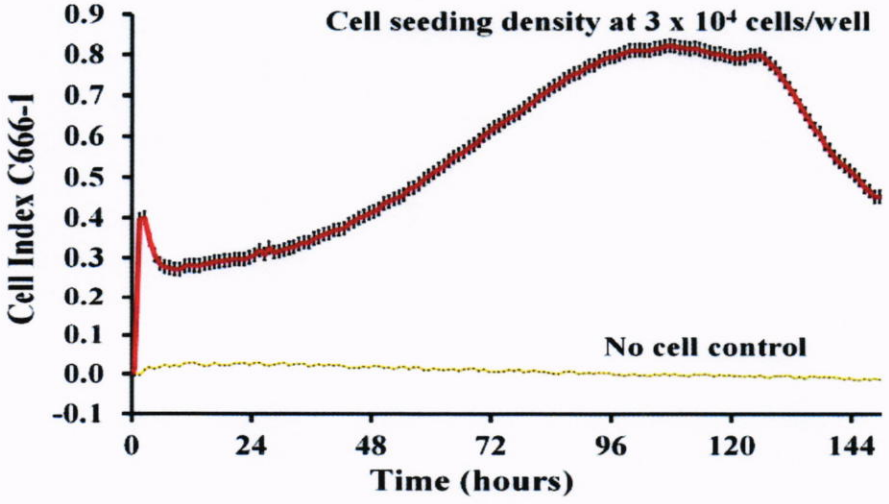
A



B



C



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4.2.1 Toxicity of DMSO on Cell Morphology and Viability

In this study, DMSO was used as a solvent for Nutlin-3. Nutlin-3 was dissolved in 99.5% DMSO which was then diluted in the culture medium. The final concentration of DMSO in each culture did not exceed 0.1%. The 0.1% DMSO-treated C666-1, NP69 and NP460 cells maintained their morphology in an elongated shape and adherent appearance throughout the treatment duration which is suggestive of proliferation (Figures 4.2 A–C). There was no significant difference in cell morphology and proliferation rate of these cell lines when compared to the respective untreated-cells, indicating that it is Nutlin-3, but not DMSO which contributes to the cytotoxic effect. All data was normalised and validated by using 0.1% DMSO-treated cells.

4.2.2 Cytotoxicity of Single Treatment Cisplatin and Nutlin-3 on Cell Morphology and Viability

C666-1 cells treated with cisplatin or Nutlin-3 were rounded in shape and detached from the culture surface. There was a slowdown in cell proliferation, where C666-1 cells became more rounded and shrunken in response to increasing concentrations of cisplatin, as well as Nutlin-3 treatment (Figure 4.2 A). The C666-1 cells which underwent further retraction were fragmented when exposed to the highest concentration of cisplatin. These observations suggest that cisplatin or Nutlin-3 can ameliorate the morphological changes as well as inhibit cell proliferation on C666-1 cells. Similar effects were observed in NP69 and NP460 cells treated with cisplatin (Figures 4.2 B–C), with NP460 cells more tolerant to cisplatin when compared to NP69 cells. Both NP69 and NP460 cells were particularly less susceptible than C666-1 cells to Nutlin-3 with less morphological alteration observed.

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Figure 4.2 (A): The morphological changes of C666-1 cells following 72 h single treatment cisplatin and Nutlin-3 as compared to 0.1% DMSO-treated and untreated cells. The images of C666-1 cells were captured using an inverted microscope at 100x magnification and saved with scale bar 100 μm.

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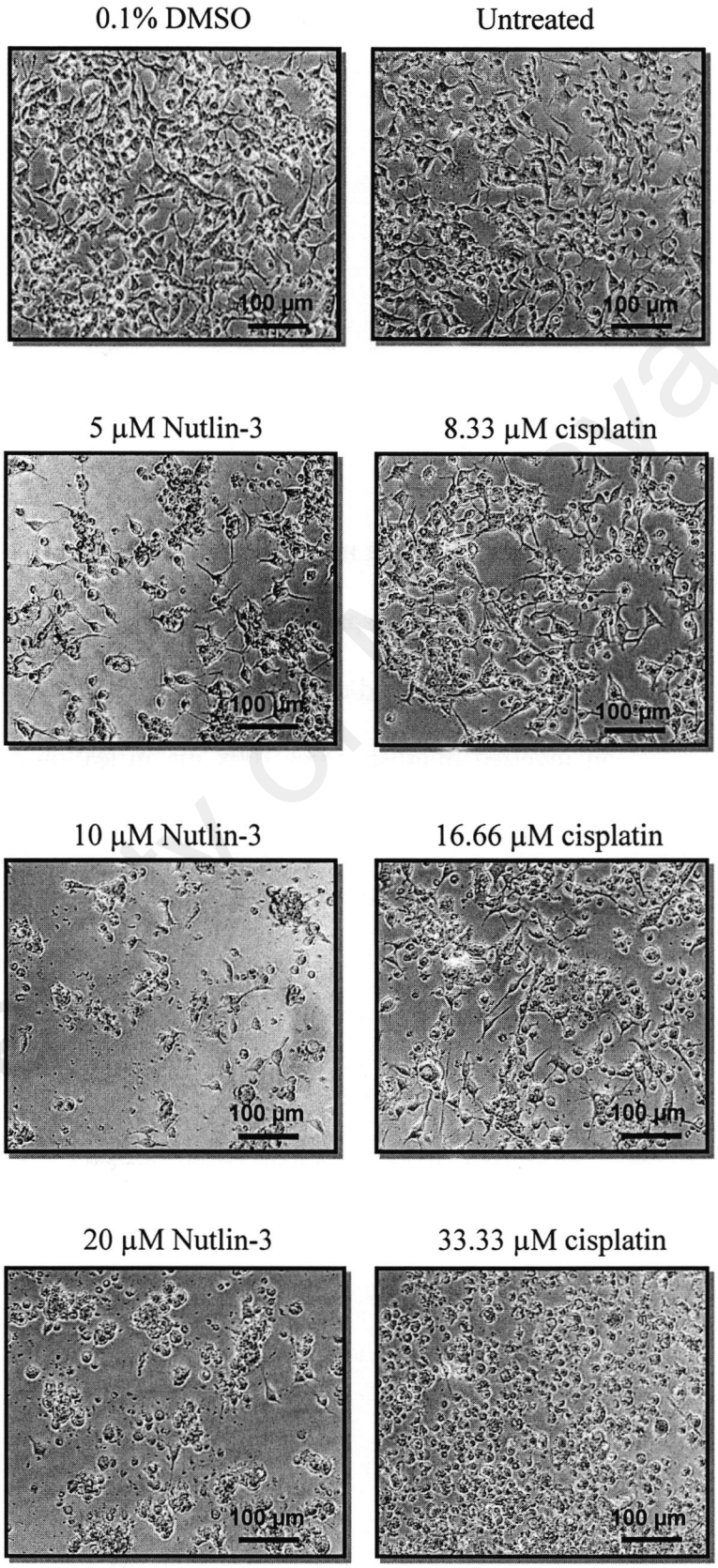

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Figure 4.2 (B): The morphological changes of NP69 cells following 72 h single treatment cisplatin and Nutlin-3 as compared to 0.1% DMSO-treated and untreated cells. The images of NP69 cells were captured using an inverted microscope at 100x magnification and saved with scale bar 100 μm.

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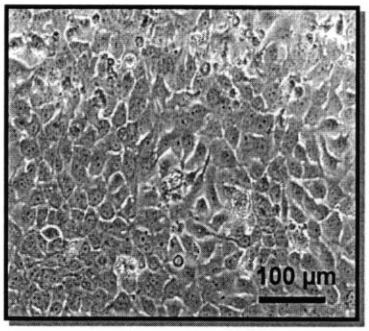
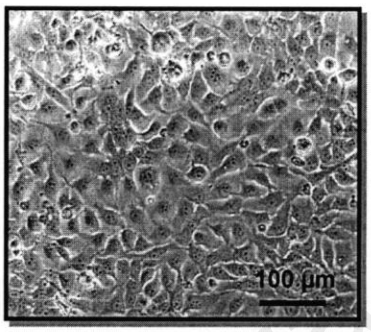
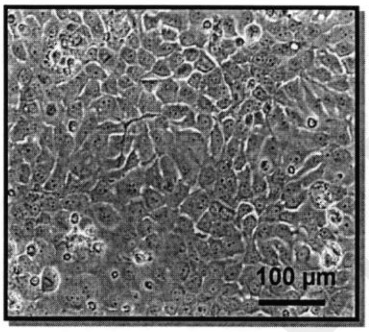
0.1% DMSO



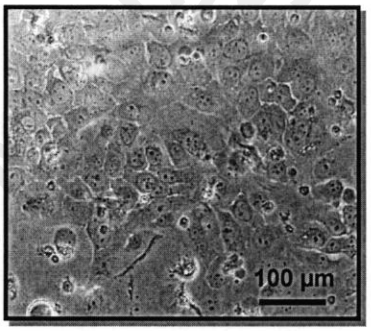
Untreated



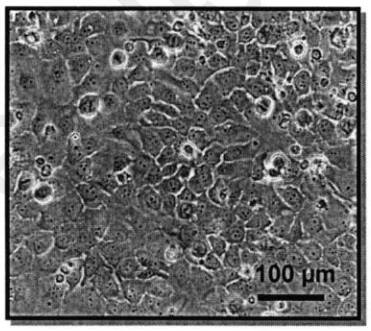
5 µM Nutlin-3



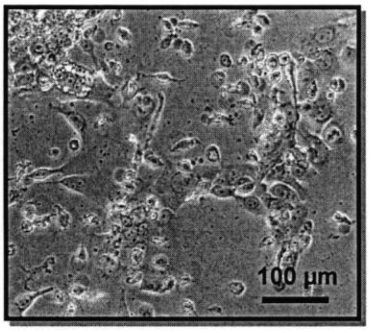
8.33 µM cisplatin



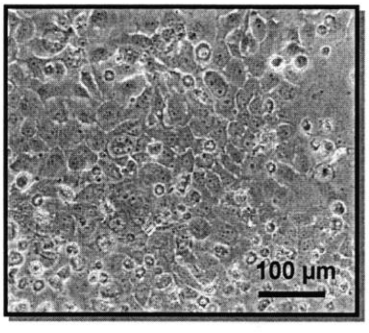
10 µM Nutlin-3



16.66 µM cisplatin



20 µM Nutlin-3



33.33 µM cisplatin

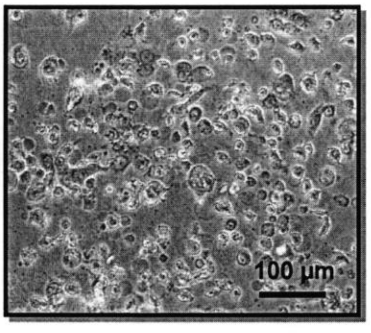


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Figure 4.2 (C): The morphological changes of NP460 cells following 72 h single treatment cisplatin and Nutlin-3 as compared to 0.1% DMSO-treated and untreated cells. The images of NP460 cells were captured using an inverted microscope at 100x magnification and saved with scale bar 100 μm.

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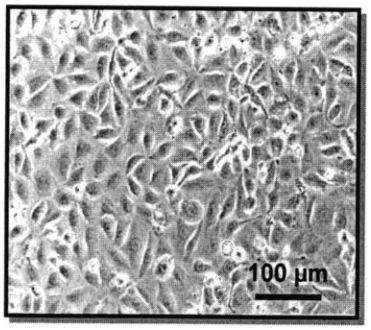
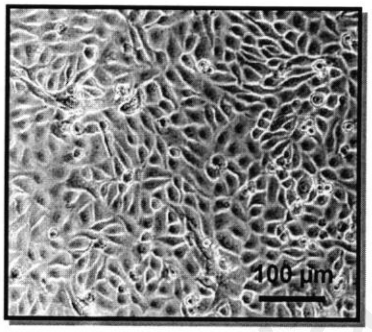
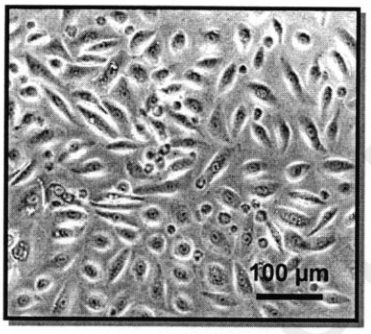
0.1% DMSO



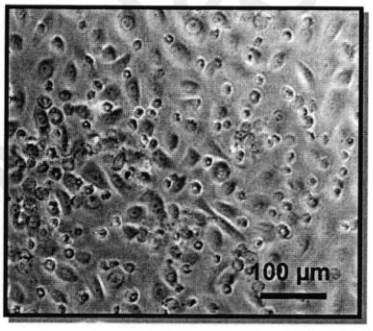
Untreated



5 µM Nutlin-3



8.33 µM cisplatin



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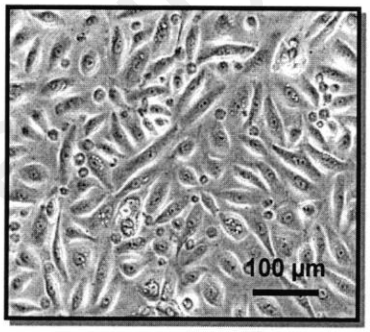
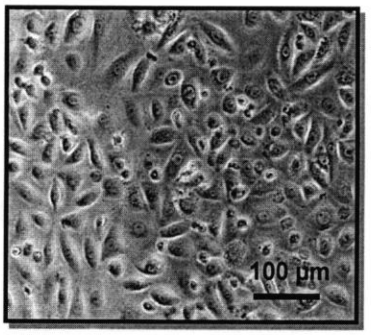


16.66 µM cisplatin



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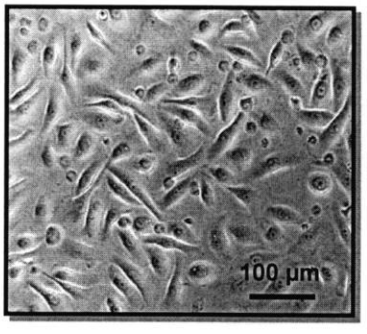
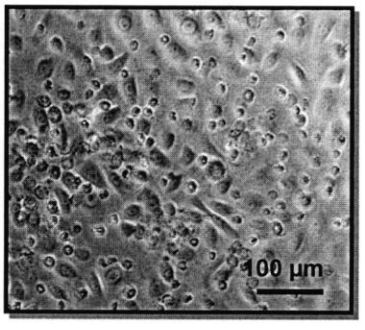


33.33 µM cisplatin



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The cytotoxicity of single agent cisplatin (Figure 4.3 A) and Nutlin-3 (Figure 4.3 B) on cell viability of C666-1, NP69 and NP460 cells was tested. The viability of NP69, NP460 and C666-1 cells decreased to 80%, 90% and 50% at 33.33 μM cisplatin treatments, respectively. Nutlin-3 inhibited C666-1 cells proliferation by 50% at 20 μM, while there was no significant cytotoxicity observed in Nutlin-3-treated NP69 and NP460 cells at a similar dose. At concentration of >20 μM, Nutlin-3 induced a dramatic growth inhibition effect on both NPC and NPE cells. The proliferation of NPE cells was completely inhibited when compared to NPC cells when the concentration of Nutlin-3 reached 40 μM. NPC and NPE cells were sensitive to cisplatin and showed clear dose dependence effect, whereas NPE cells were less sensitive than NPC cells to Nutlin-3. The IC_{50} values (Table 4.2) indicated that cisplatin was more cytotoxic against NPE compared to NPC cells.

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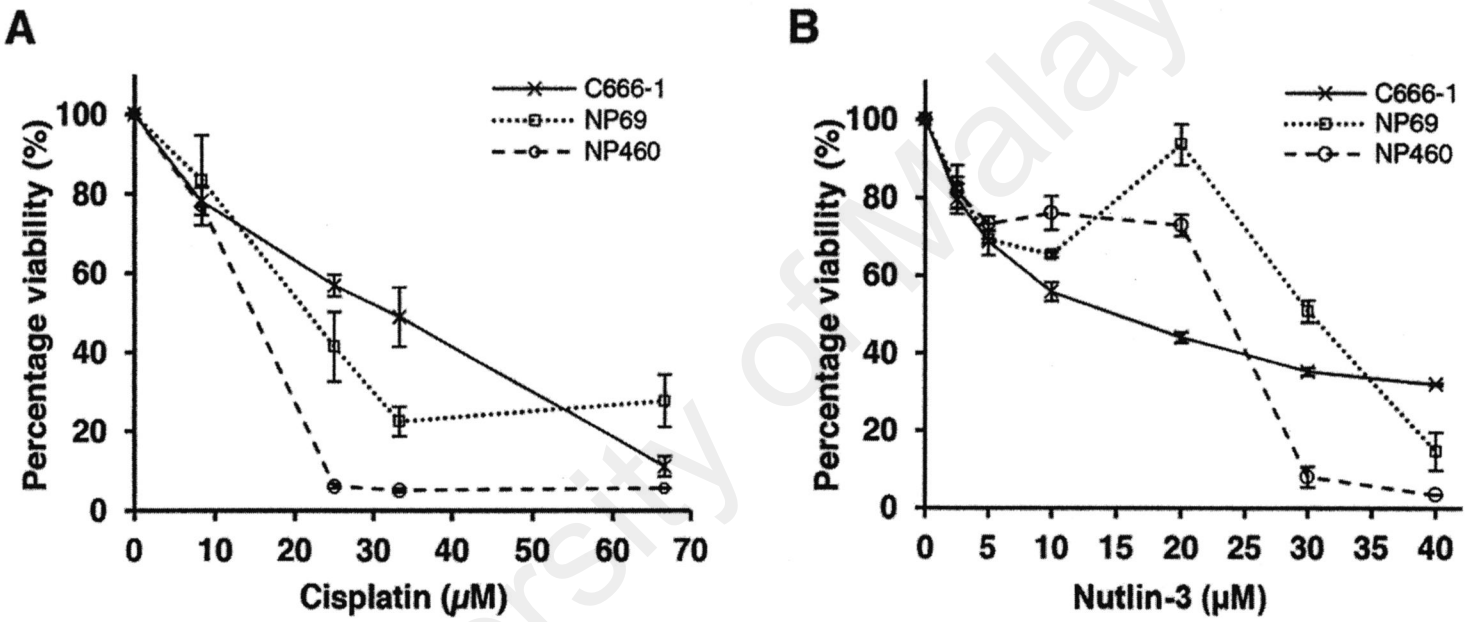

Figure 4.3: Growth-inhibitory effects of cisplatin and Nutlin-3 on NPC (C666-1) and NPE (NP69, NP460) cells. Cells were treated with increasing concentrations of (**A**) cisplatin (0–66.64 μM) or (**B**) Nutlin-3 (0–40 μM) for 72 h. Cell viability was determined by MTS viability assay. The mean percentage of cell viability relative to untreated and 0.1% DMSO-treated controls are shown.

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Table 4.2: Sensitivity of NPC and NPE cells to single treatment of cisplatin and Nutlin-3 as indicated by IC_{50} ± SD values. Representative IC_{50} ± SD values are the mean of three independent experiments with nine data points

Cell Line	Cell Type	IC_{50} value ± SD (μM)	
		Cisplatin	Nutlin-3
NP69	Immortalized human NPE, SV40 infection	19.31 ± 4.75	31.69 ± 2.54
NP460	hTERT human NPE, telomerase transfection	14.18 ± 2.97	22.85 ± 1.18
C666-1	Epithelial, undifferentiated carcinoma	32.07 ± 4.18	19.95 ± 8.93

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4.2.3 Cytotoxicity of Cisplatin and Nutlin-3 Treatment Combination on Cell Viability

The effect of Nutlin-3 in combination with cisplatin on C666-1 cells was investigated. Cisplatin alone inhibited the growth of C666-1 cells in a dose-dependent manner with IC_{50} value of 26.55 ± 6.15 μM (Table 4.3). When C666-1 cells were treated with a combination of cisplatin (0–100 μM) and a fixed concentration of 10 μM Nutlin-3, the IC_{50} value was markedly reduced to 3.67 ± 0.88 μM (Figure 4.4 A) indicating that Nutlin-3 enhanced cisplatin-induced cells death in C666-1 cells. Similarly, when the cells were treated with a combination of Nutlin-3 (0–30 μM) and a fixed concentration of 33.33 μM cisplatin, the IC_{50} value Nutlin-3 was dramatically decreased from 10.23 ± 0.87 μM to 0.86 ± 0.13 μM (Figure 4.4 B), suggesting that cisplatin combined with Nutlin-3 was more effective than when each agent was used separately. These findings provide an early indication that Nutlin-3 sensitises C666-1 cells to the cytotoxic effects of cisplatin.

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Table 4.3: Sensitivity of NPC C666-1 cells to combination treatment of cisplatin and Nutlin-3 as indicated by IC_{50} ± SD values. Representative IC_{50} ± SD values are the mean of three independent experiments with nine data points

Treatments	IC_{50} values (μM)
Cisplatin alone (0–100 μM)	26.55 ± 6.15
Cisplatin + Nutlin-3 (10 μM)	3.67 ± 0.88
Nutlin-3 alone (0–30 μM)	10.23 ± 0.87
Nutlin-3 + cisplatin (33.33 μM)	0.86 ± 0.13

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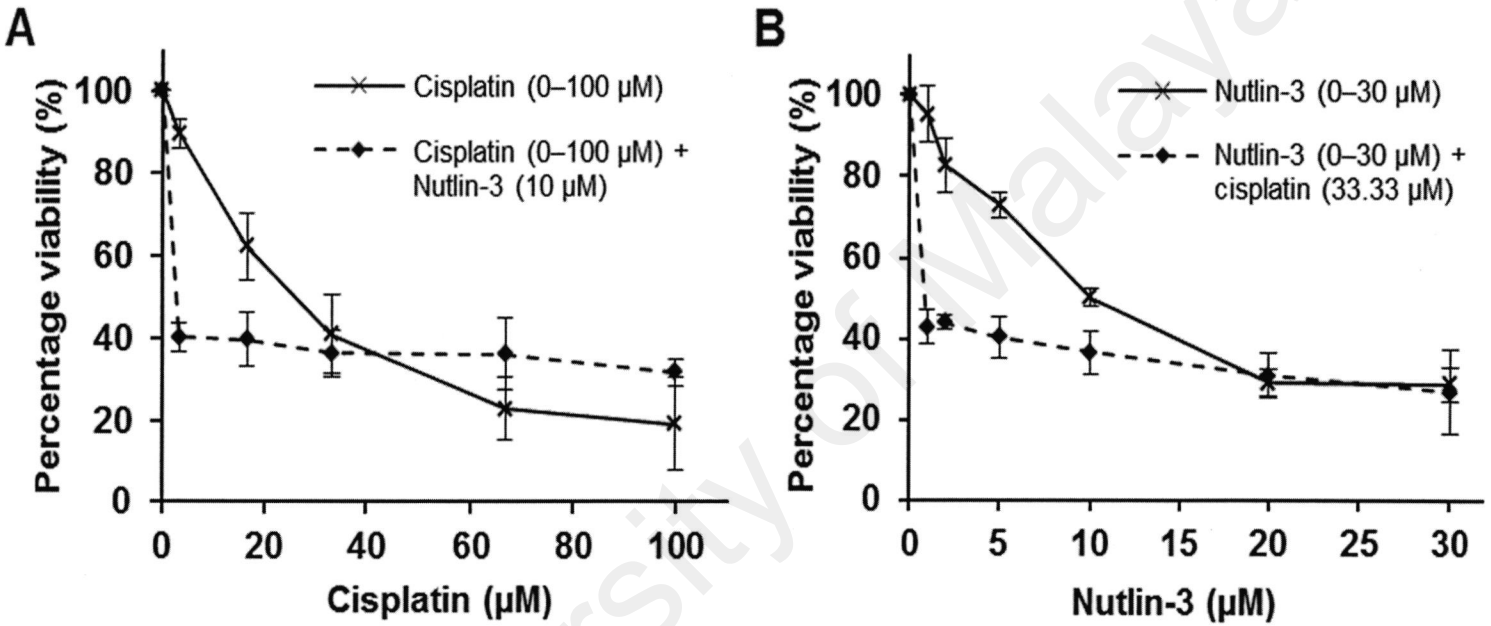

Figure 4.4: Growth-inhibitory effects of cisplatin and Nutlin-3 treatment combination on NPC cells. Nutlin-3 in combination with cisplatin inhibits the cell viability of C666-1 cells. The cell viability in response to (**A**) cisplatin alone (0–100 μM) and in combination with 10 μM Nutlin-3; and (**B**) Nutlin-3 alone (0–30 μM) and in combination with 33.33 μM cisplatin were assayed by MTS viability assay at 48 h treatment. The graphs shown are representative mean percentages of cell viability relative to untreated and 0.1% DMSO-treated controls of three independent experiments with nine data points.

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4.2.4 Effects of Nutlin-3 on Anchorage-independent Growth of NPC Cells in Soft Agar

The effects of Nutlin-3 in combination with cisplatin on the tumorigenicity of C666-1 cells were investigated by determining the efficiency of anchorage-independent colony formation using soft agar colony formation assay. The 0.1% DMSO-treated and untreated C666-1 cells display anchorage-independent growth as evidenced by the ability to proliferate and develop colonies in soft media in which they float freely, an indication of tumourigenicity (Figure 4.5). The highest number of colonies was observed in untreated and DMSO-treated C666-1 cells. Cisplatin (33.33 μM) and Nutlin-3 (10 μM) significantly reduced the number of colonies formed when administered alone relative to the observations in untreated and DMSO-treated controls. When the two treatments were combined, the number of colonies formed was markedly reduced and the sizes of the colonies were also significantly smaller (Figure 4.6). These findings further support the earlier observation that Nutlin-3 sensitises C666-1 cells to the cytotoxic effect of cisplatin and suppresses colony formation.

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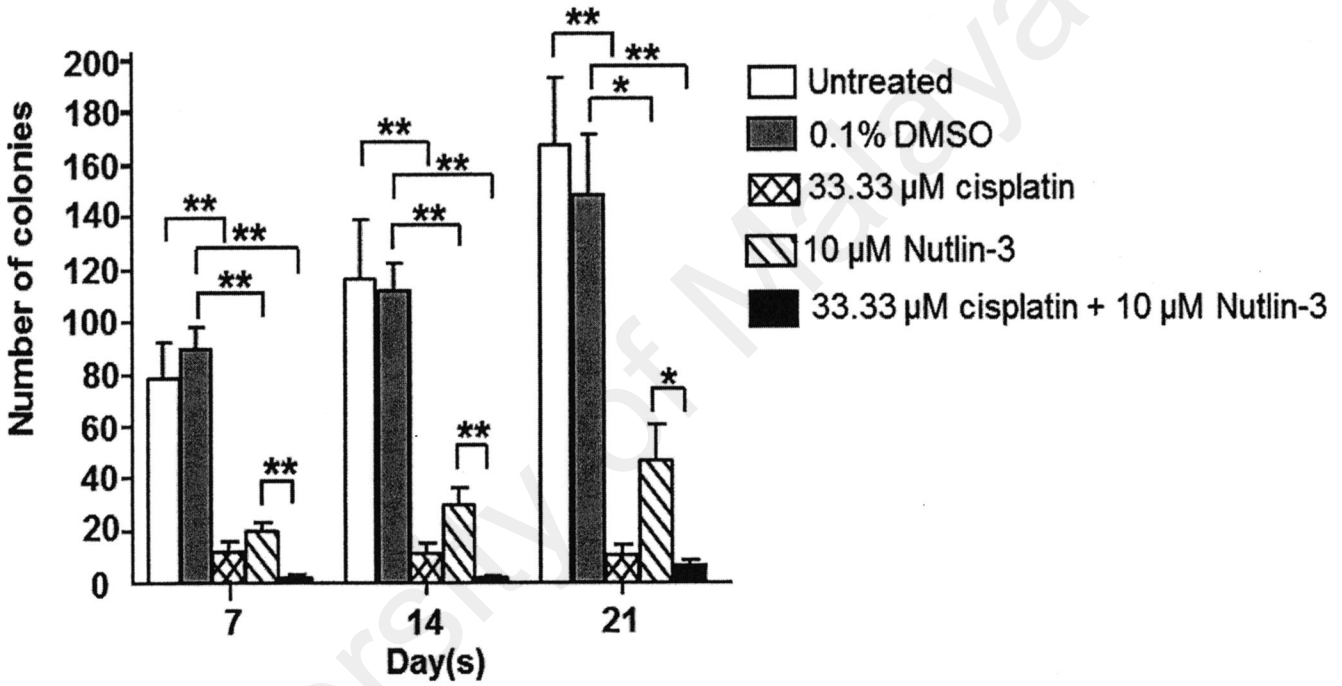

Figure 4.5: The effects of Nutlin-3 alone and in combination with cisplatin on colony formation of C666-1 cells. The plot shown is representative of two independent experiments carried out in triplicates. The untreated and 0.1% DMSO-treated cells were included as controls for cisplatin and Nutlin-3 treatments, respectively. Statistical analysis was performed to compare the difference in number of colonies scored at Days 7, 14 and 21 compared to the controls. * and ** indicates statistically significant different (p<0.05, Student's t-test) and (p<0.005, Student's t-test), respectively.

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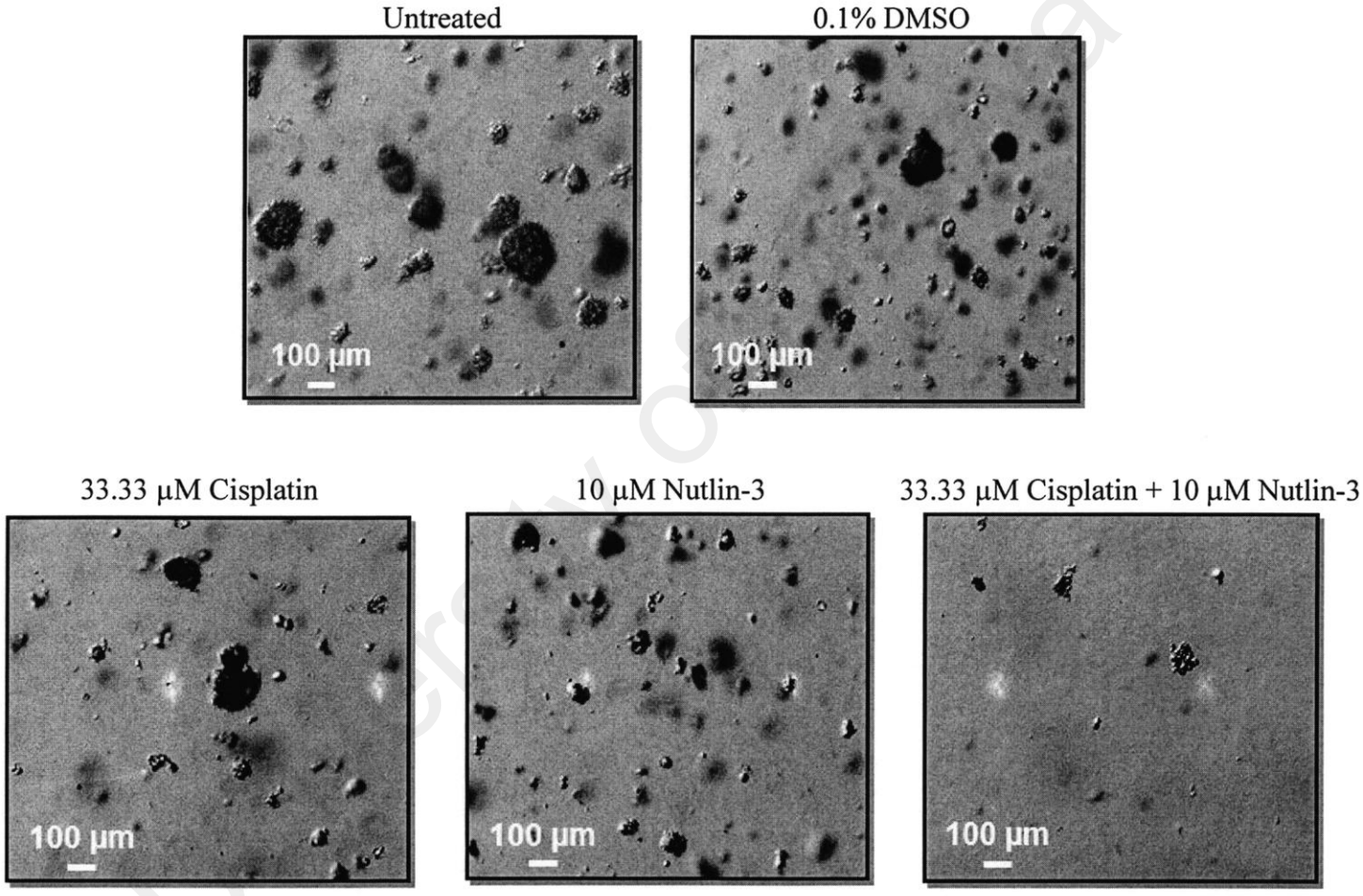

Figure 4.6: The morphological appearances of C666-1 colonies in the soft agar cultures. The image of the colonies was captured on day 21 by stereo microscope at magnification of 250x (scale bar 100 µm).

4.3 Effects of Nutlin-3 on the p53 Pathway

4.3.1 Effects of Nutlin-3 on p53 Pathway in wt p53 NPC Cells

The effects of Nutlin-3 on the disruption of p53-Mdm2 interaction were investigated on NPC C666-1 and HK1 cells harbouring wt and mutant p53, respectively. The investigation was performed in parallel with colorectal (HCT116) and breast (MDA-MB-231) cancer cells harbouring wt and mutant p53, respectively. Activation of p53 pathway was induced following 24 h treatment with 10 μM Nutlin-3. The expression of cellular proteins p53, p21 and Mdm2 are shown in Figure 4.7. Upon treatment of C666-1 or HCT116 cells with Nutlin-3, p53 was activated which significantly induced the expression of p21 protein and, to a lesser extent, Mdm2 protein. The effects of Nutlin-3 were stronger in cells bearing wt p53 when compared to cells with mutated p53 (HK1 and MDA-MB-231). The findings suggest that Nutlin-3 activates the p53 pathway and induces the up-regulation of p53, p21 and Mdm2 in cells bearing wt p53, which is in agreement with that of previous studies suggesting that the disruption of p53-Mdm2 interaction to reactivate p53 pathway by Nutlin-3 is restricted to wt p53 (Vassilev *et al.*, 2004).

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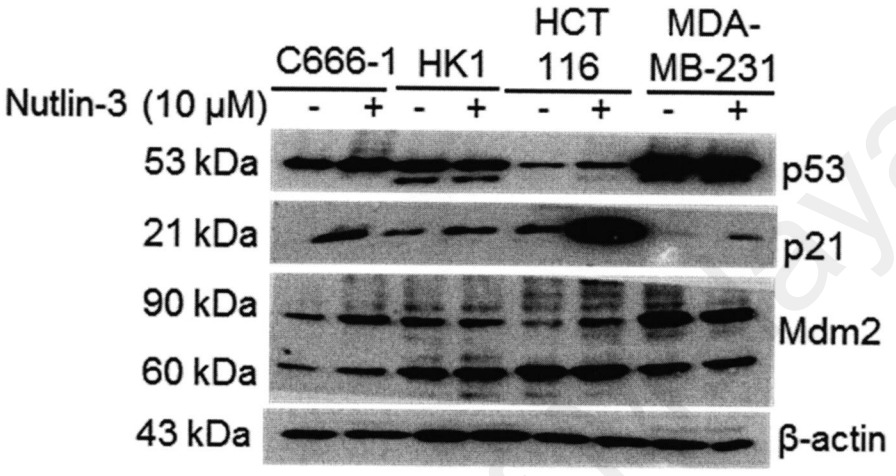

Figure 4.7: Nutlin-3 activates p53 pathway NPC cells in a p53-dependent manner. The expression of p53-related proteins in cancer cells bearing wt p53 (C666-1, HCT116) and mutant p53 (HK1, MDA-MB-231) following treatment with 10 μM Nutlin-3 (+) or 0.1% DMSO (-) for 24 h are shown. β-actin is included as loading control. Nutlin-3 induces the up-regulation of p53, p21 and Mdm2 proteins in C666-1 and HCT116 cells bearing wt p53. C666-1 and HK1, NPC cell lines; HCT116, colorectal carcinoma cell line; MDA-MB-231, breast cancer cell line.

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4.3.2 Effects of Nutlin-3 on p53 Knockdown NPC Cells

4.3.2.1 Establishment of p53 Knockdown in C666-1 NPC Cells

To verify that the effects of Nutlin-3 are mediated via the p53 pathway, p53 gene knockdown in C666-1 cells was performed using four different lentiviral-based shRNA constructs (p53si-E1, -E2, -C12 or -D3). Fluorescent emission signal detection in Vector-GFP was used to measure transfection efficiency and optimise shRNA delivery (Figure 4.8, h). The efficiency of knockdown was verified by examining the p53 protein expression level as showed in Figure 4.9; Vector-pLKO and NS were used as controls. No cytopathic effect was observed in lenti-shΔp53 C666-1 cells following lentiviral infection as compared to parental C666-1 cells. The cells transduced with p53si-E2 and p53si-D3 constructs had 70% ($p<0.05$, Student's t-test) and 90% ($p<0.005$, Student's t-test) knockdown of p53 protein expression, respectively; whereas, the controls Vector-pLKO and NS had unaltered p53 protein expression. The order of efficacy knockdown was determined to be p53si-D3 > p53si-E2 > p53si-C12 > p53si-E1.

4.3.2.2 Effects of Nutlin-3 on p53 Pathway in p53 Knockdown NPC Cells

The effects of Nutlin-3 on the expression of p53-related proteins following the knockdown of p53 protein in the lenti-shΔp53-transduced cells were studied. When the parental C666-1 cells were exposed to 10 μM Nutlin-3, the increased expression of p53, p21 and Mdm2 were observed in comparison to the untreated-parental C666-1 cells and controls Vector-pLKO and NS (Figure 4.10). However, these effects were dramatically inhibited in p53 knockdown C666-1 cells transduced with p53si-D3 or p53si-E2 constructs, indicating that the effects of Nutlin-3 on the expression of p53-related proteins in NPC cells are p53-dependent.

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Figure 4.8: The morphological appearances of lenti-shΔp53 C666-1 cells and the GFP signal at 96 h following lentiviral infection. The representative images of the lenti-shΔp53 C666-1 cells (a)–(g) were captured using an inverted microscope at 100x magnification and saved with scale bar of 100 μm. The Vector-GFP (h) illustrates the fluorescent emission detected in C666-1 cells transfected with vector-green fluorescent protein. The green fluorescent signal was detected by an Inverted Live Cell Imaging system.

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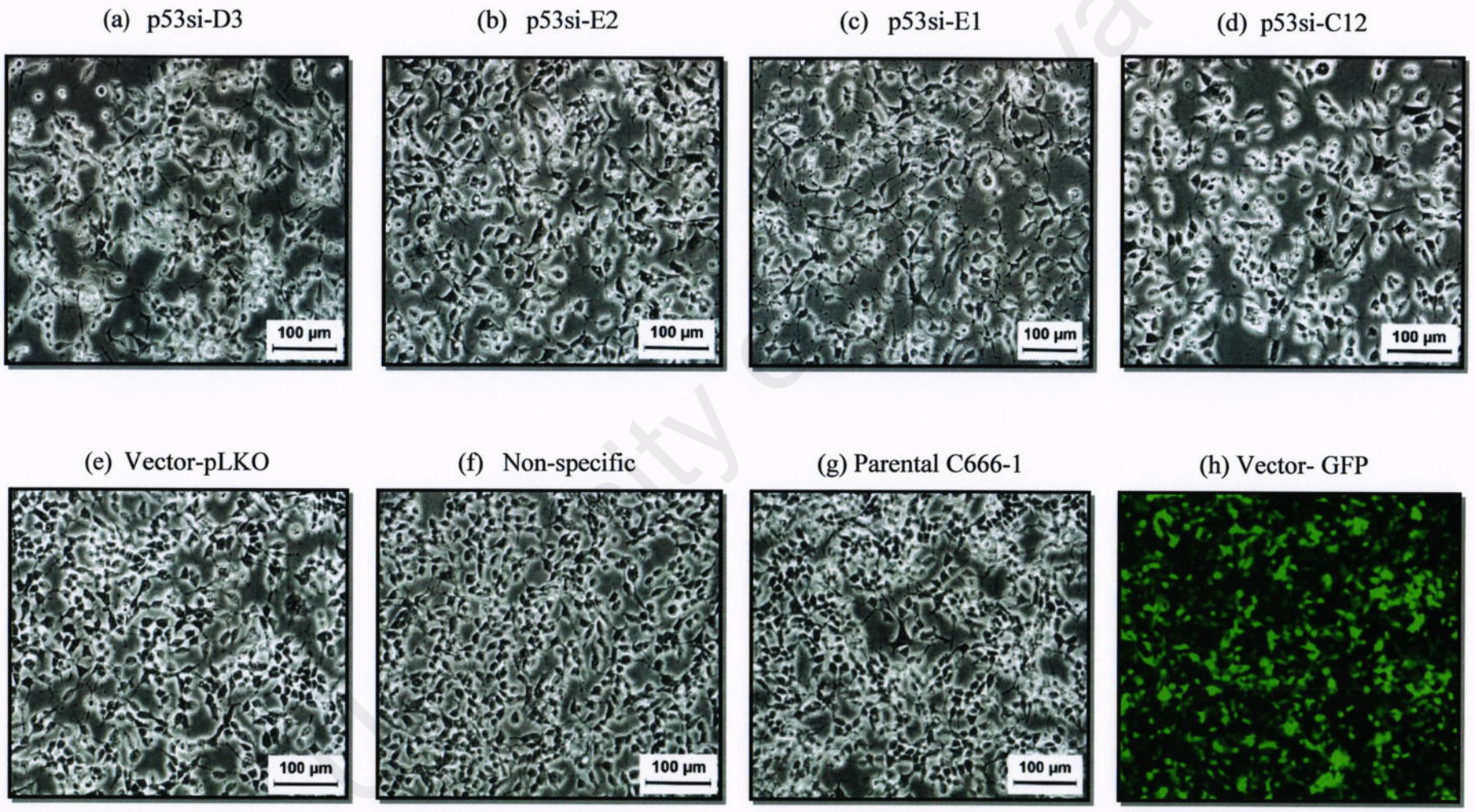
(a) p53si-D3
(b) p53si-E2
(c) p53si-E1
(d) p53si-C12
(e) Vector-pLKO
(f) Non-specific
(g) Parental C666-1
(h) Vector- GFP
100 µm

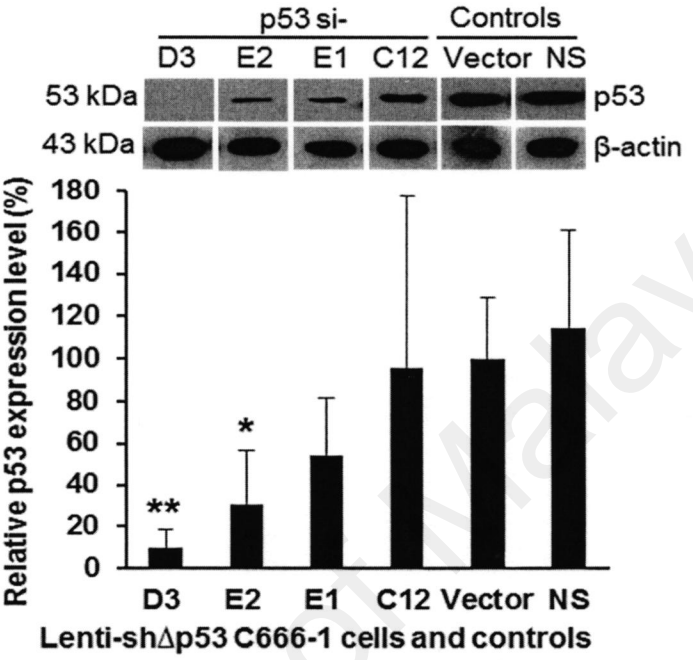

Figure 4.9: Establishment of p53 knockdown in C666-1 cells. The p53-knocked down C666-1 cell sublines (lenti-shΔp53 C666-1) were generated using lentiviral-based shRNA constructs p53si-E1, -E2, -C12 or -D3 in parallel with controls Vector and NS. The efficiency of knock down was verified at 96 h post transduction. The plot shown is representative of three independent experiments. The mean percentage of relative p53 expression level was obtained by densitometry analysis when normalised to β-actin. * and ** indicates statistically significant different (p<0.05, Student's t-test) and (p<0.005, Student's t-test), respectively. Among the four constructs, the p53 protein level was most significantly reduced by p53si-D3 or p53si-E2. Vector, vector-pLKO; NS, non-specific.

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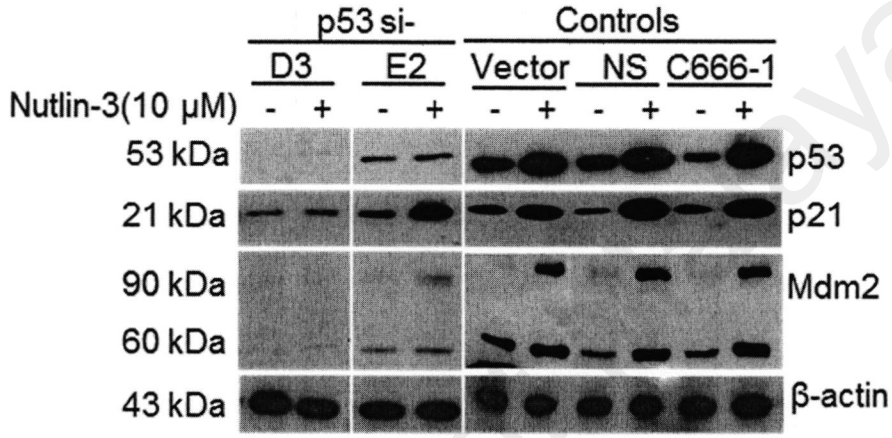

Figure 4.10: The effects of Nutlin-3 on the expression of p53, p21 and Mdm2 proteins in lenti-shΔp53 C666-1 cells. The lenti-shΔp53 C666-1 and control cells were treated with 10 μM Nutlin-3 for 24 h prior to analysis. β-actin is included as loading control and verifiable for equal loading of proteins. The attenuation of the activation of p53 pathway was evident in cells transduced with p53si-D3 or p53si-E2 when compared to the controls. Vector, vector-pLKO; NS, non-specific.

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4.3.2.3 Cytotoxicity of Nutlin-3 on Cell Viability in p53 Knockdown NPC Cells

Next, the cytotoxicity of Nutlin-3 on the growth and viability of lenti-shΔp53 C666-1 cells was tested. The percentages of viability relative to controls vector-PLKO and NS following treatment with Nutlin-3 are shown in Figure 4.11. Cells with p53 knocked down were found less sensitive to Nutlin-3 in comparison to controls. The concentration of Nutlin-3 required to induce 50% cell death in the lenti-shΔp53 C666-1 cells p53si-E2 or D3 was determined to be > 40 μM. These findings indicate that the reduction of lenti-shΔp53 C666-1 cells sensitivity to Nutlin-3 is associated with p53-knock down. Taken together, these findings suggest that Nutlin-3 activates the p53 pathway and exerts its cytotoxic effects on NPC cells in a p53-dependent manner.

4.4 Investigation on the Plausible Mechanisms of Cell Death on NPC Cells

4.4.1 Effects of Nutlin-3 on Apoptosis in Cisplatin-treated NPC Cells

The combination treatment of cisplatin and Nutlin-3 effectively impaired cell viability and markedly suppressed the tumourigenicity of C666-1 cells as shown in sections 4.2.3 and 4.2.4. The effects of Nutlin-3 in combination with cisplatin on induction of apoptosis were determined using high content imaging of Annexin V/propidium iodide-stained cells (Figure 4.12). The apoptotic cells scores are shown in Figure 4.13. Very low percentage of apoptotic cells was detected in the untreated and 0.1% DMSO-treated cells at less than 5% following both 48 and 72 h post treatment. Nutlin-3 alone exerted less apoptotic effects on C666-1 cells, 5% at 48 h treatment; the Annexin V-stained cells increased by 5% at 72 h treatment. Treatment of C666-1 cells with cisplatin resulted in moderate apoptotic cell death, 10% and 20%, for 48 and 72 h, respectively. Apoptosis increased significantly in cells treated with both cisplatin and Nutlin-3; increased from 5% (untreated) to 20% (48 h) and 35% (72 h) indicating that Nutlin-3 sensitised C666-1 cells to cisplatin and enhanced apoptotic cell death.

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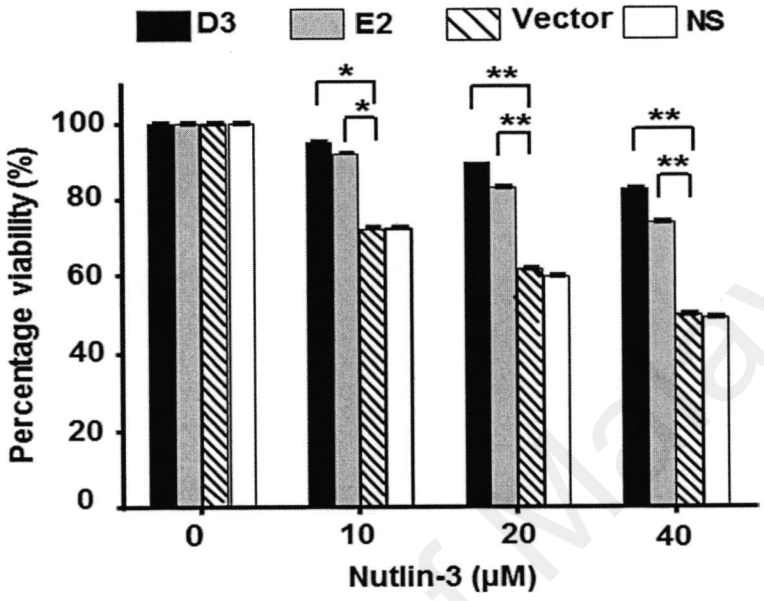

Figure 4.11: Growth-inhibitory effects of Nutlin-3 on lenti-shΔp53 C666-1 cells. Cell viability responses to 0–40 μM Nutlin-3 at 72 h treatment was quantified by MTS viability assay. The mean percentage of cell viability relative to 0.1% DMSO-treated vehicle control is shown for the lenti-shΔp53 C666-1 and controls Vector-pLKO and NS. The plot shown is representative of mean values of three independent experiments with nine data points. * and ** indicates statistically significant different (p<0.05, Student's t-test) and (p<0.005, Student's t-test), respectively. D3, p53si-D3; E2, p53si-E2; Vector, vector-pLKO; NS, non-specific.

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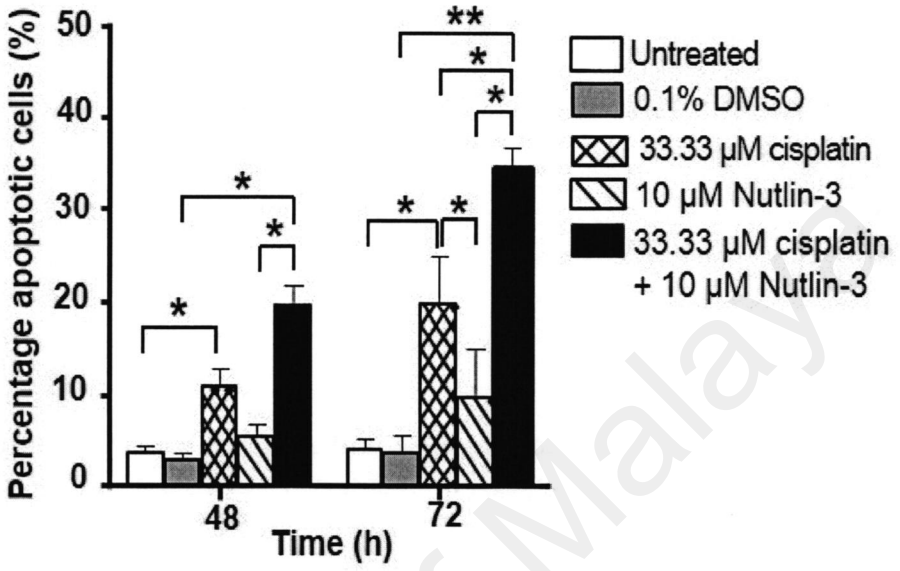

Figure 4.12: High content analysis of Annexin V-FITC/ PI-stained cells to measure apoptosis induced by cisplatin and/or Nutlin-3 in C666-1 cells. The cells were treated with 33.33 µM cisplatin and/or 10 µM Nutlin-3 for 48 and 72 h prior to analysis. The untreated and 0.1% DMSO-treated cells served as non-treated controls for cisplatin and Nutlin-3, respectively. Statistical analysis was performed to compare the difference in the apoptotic cell scores in cisplatin-treated and/or Nutlin-3-treated cells when compared to the controls. * and ** indicates statistically significant difference ($p<0.05$, Student's t-test) and ($p<0.005$, Student's t-test), respectively. FITC, fluorescein isothiocyanate; PI, propidium iodide.

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Figure 4.13: High content images of Annexin V-FITC/ PI-stained C666-1 cells induced by cisplatin and/or Nutlin-3. Representative high content images of C666-1 cells stained with Annexin V-FITC for apoptosis (**green**), PI for viability (**red**) and Hoechst 33342 as counterstained (**blue**) are shown. Imaging was conducted at 72 h post-treatment at magnification of 20x. Annexin V (+)/ PI (-) and Annexin V (+)/ PI (+) cells were defined as apoptotic cells. FITC, fluorescein isothiocyanate; PI, propidium iodide.

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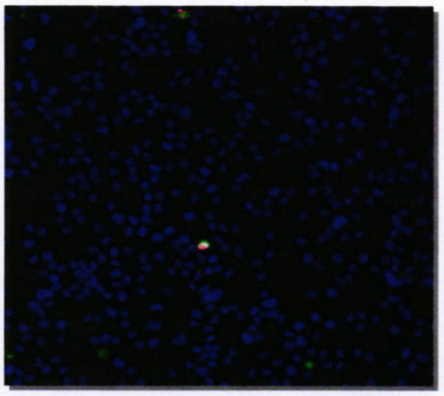
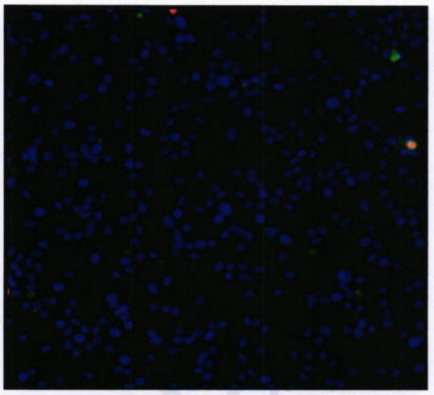
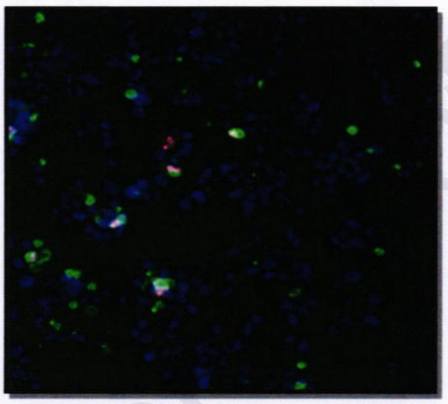
Untreated



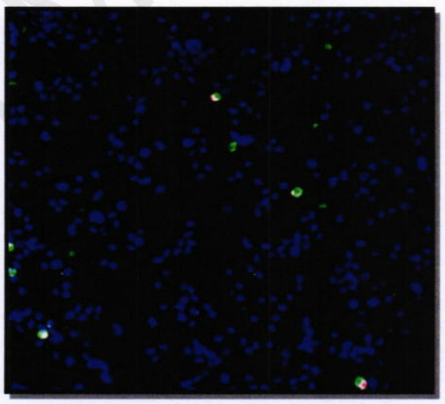
0.1% DMSO



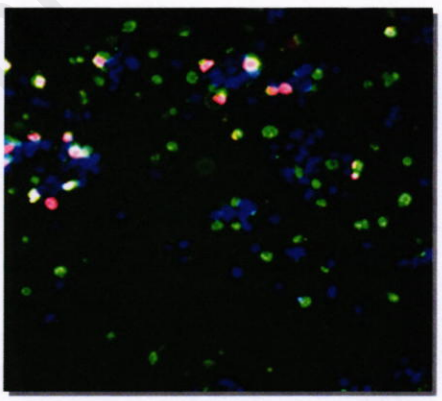
33.33 μM Cisplatin

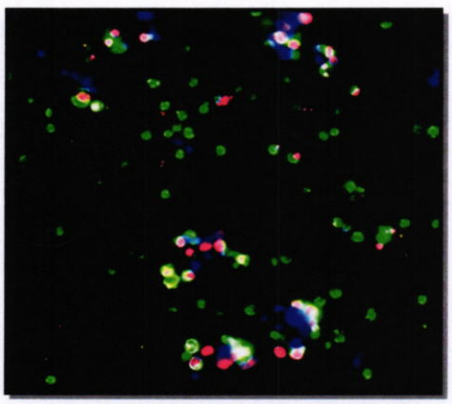


10 μM Nutlin-3



33.33 μM Cisplatin + 10 μM Nutlin-3





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4.4.2 Effects of Nutlin-3 on the Activation of Apoptosis-related Protein Expression in NPC Cells

This study has shown that Nutlin-3 enhanced cisplatin-induced apoptotic cell death in C666-1 cells (Figure 4.12); hence the effects of Nutlin-3 on the activation of p53-mediated pro-apoptotic genes (Figure 4.14) were further investigated. Treatment with Nutlin-3 or cisplatin alone upregulated p53 and Mdm2 proteins expression when compared to DMSO-treated control. Additionally, treatment with Nutlin-3 alone had upregulated BAX and PUMA proteins expression. Similarly, the expressions of p53, BAX and PUMA were further enhanced by using a combination therapy of cisplatin and Nutlin-3. Furthermore, the cleaved PARP level detected in the cells treated with the combination drugs was consistent with apoptosis. Taken together, these results suggest that Nutlin-3 sensitises C666-1 cells to cisplatin-induced apoptosis by modulating pro-apoptotic targets BAX and PUMA.

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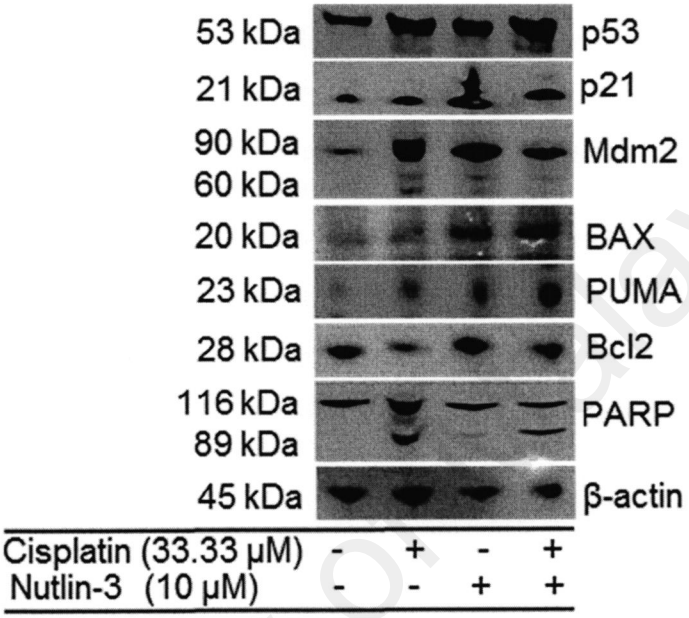

Figure 4.14: Nutlin-3 activates the expression of apoptosis-related proteins in cisplatin-treated C666-1 cells. C666-1 cells were treated with cisplatin and/or Nutlin-3 (+) or 0.1% DMSO (-) for 24 h prior to analysis. The level of p53 target apoptosis-related proteins is shown. β-actin was probed to monitor protein loading. Treatment of C666-1 cells with Nutlin-3 resulted in induction of p53 target apoptosis-related proteins p21, Mdm2, BAX and PUMA when compared to vehicle control.

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4.5 Effects of Nutlin-3 on the Emergence of p53 Mutations in NPC Cells

C666-1 cells were treated with various concentrations of Nutlin-3 for extended periods to assay emergence of Nutlin-3-resistant C666-1 cells from adaptation to Nutlin-3 treatments (Figure 3.5). The attained Nutlin-3-resistant C666-1 cells were investigated to determine whether the emergence of Nutlin-3-resistance is associated with p53 gene alterations as a result of long-term treatment effects of Nutlin-3. The Nutlin-3-treated NPC sublines showed a doubling in IC_{50} value to Nutlin-3 when compared to parental C666-1 cells, suggesting that C666-1 cells developed relative resistance to Nutlin-3 (Figure 4.15). Next, the Nutlin-3-resistant sublines were investigated for p53 mutation status. The p53 gene sequence spanning exons 2 to 11 of the investigated sublines was found to be free of mutation compared to parental C666-1 cells (Table 4.4). These results indicate that, while extended treatment of NPC cells with Nutlin-3 resulted in decreased sensitivity to Nutlin-3, it is not associated with the emergence of p53 mutations at the investigated dose and duration.

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Figure 4.15: Extended treatment with Nutlin-3 resulted in reduced sensitivity without emergence of p53 mutation. Sensitivity of Nutlin-3-adapted C666-1 sublines to Nutlin-3 indicated by the concentration that inhibits cell viability by 50% (IC_{50}) at 72 h treatment as quantified by MTS viability assay. The plot shown is representative mean IC_{50} values of three independent experiments carried out in triplicate. * and ** indicates statistically significant different ($p<0.05$, Student's t-test) and ($p<0.005$, Student's t-test), respectively. R, resistance index indicates the degree of acquired resistance. ($\mathcal{X}$), Nutlin-3-adapted C666-1 sublines; R, resistance index indicates the degree of acquired resistance.

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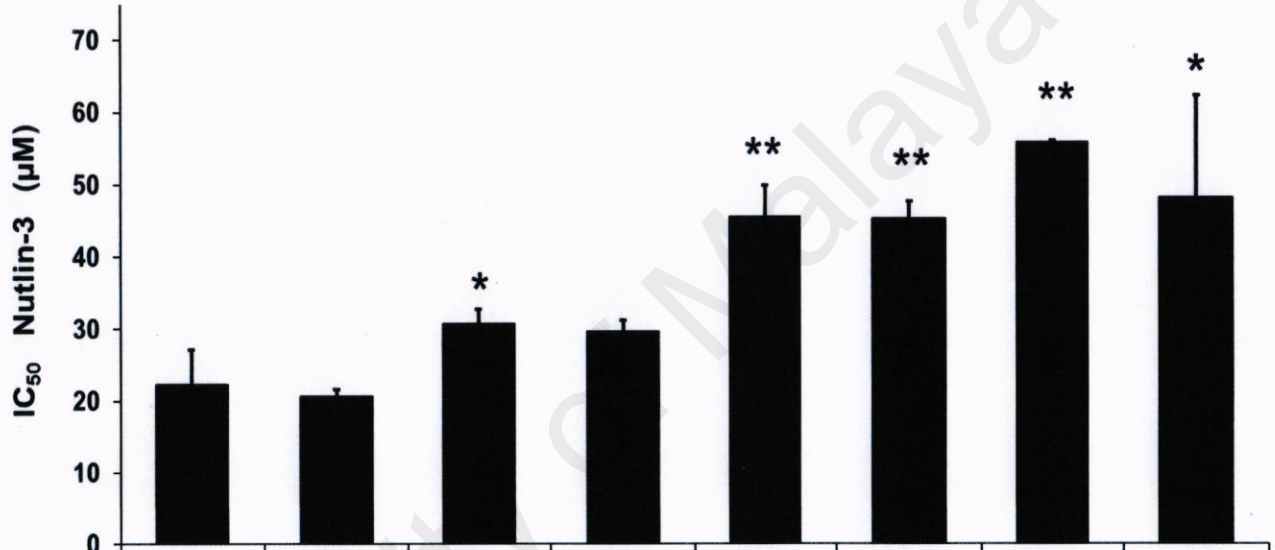

Nutlin-3-adapted sublines (χ)	A	B	C	D	E	F	G	H
Number of passages at 0 μM	29	0	0	0	0	0	0	0
Number of passages at 10 μM	0	7	14	21	15	15	15	15
Number of passages at 20 μM	0	0	0	0	7	14	21	7
Number of passages at 40 μM	0	0	0	0	0	0	0	7
Total passages	29	7	14	21	22	29	36	29
IC_{50} Nutlin-3 for χ	22.28±4.86	20.63±0.95	30.74±2.06	29.76±1.52	45.56±4.32	45.35±2.27	55.90±0.14	48.22±14.13
R value of χ	1.00	0.93	1.38	1.34	2.04	2.04	2.51	2.16

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Table 4.4: Summary of p53 mutation status of exons 2nd through 11th of Nutlin-3-adapted C666-1 sublines

Exons p53	CDS	Nutlin-3-adapted C666-1 sublines		
		A	D	H
2	11717..11790	Nm	Nm	Nm
3 + 4	11906..11927 12021..12299	Nm †	Nm †	Nm †
5	13055..13238	Nm	Nm	Nm
6	13320..13432	Nm	Nm	Nm
7	14000..14109	Nm	Nm	Nm
8	14452..14588	Nm	Nm	Nm
9	14681..14754	Nm	Nm	Nm
10	17572..17678	Nm	Nm	Nm
11	18599..18680	Nm	Nm	Nm

†, a base change of C**G**C to C**C**C in exon 4th at 12139bp with no mutation detected; CDS, coding DNA sequences; Nm, No mutation detected; **A**, C666-1/wt; **D**, 21-passages with 10 µM Nutlin-3 ($N^{+21/10}$); **H**, 15-passages with 10 µM plus 7-passages with 20 µM plus 7-passages with 40 µM Nutlin-3 ($N^{+15/10}N^{+7/20}N^{+7/40}$).

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CHAPTER 5: DISCUSSION

The margin of standard therapy approach to treat the most radiocurable NPC is still dependent on concurrent megavoltage radiotherapy (Lee *et al.*, 2012a; 2012b; Phua *et al.*, 2013) despite the availability and application of molecular-based techniques. The use of cisplatin-based agent integrated into radiotherapy approach for advanced and locoregional NPC is recommended to radiosensitise targeted tumour and improve radiotherapy results. Although cisplatin is one of the most potent chemotherapy agents, its chemotherapeutic efficacy is limited by its severe irreversible adverse effects such as nephrotoxicity, ototoxicity and neurotoxicity as well as induction of mitochondrial reactive oxygen species which is correlated with metabolic dysfunctions (Choi *et al.*, 2015). The combination of radio-chemo therapies tends to cause more severe adverse effects (Anniko and Sobin, 1986; O'Sullivan, 2007; Wei and Sham, 2005). The emergence of chemo-resistance is a major limitation of cisplatin (Siddik, 2003); hence the challenge is in reducing undesirable complications and improving the treatment outcome of locoregionally advanced NPC. NPC is especially responsive to cisplatin; cisplatin-based agent combinations with more than a single chemotherapy agent are more effective than non-cisplatin-based combinations (Lee *et al.*, 2012a; 2012b). It has been reported that cisplatin induces apoptosis via activation of p53 gene (Wang *et al.*, 2006). Hence, the impact of cisplatin-induced cytotoxicity on NPE and NPC cells has been taken into consideration in this study.

Several strategies have been adopted for restoring tumour suppressor gene in tumours such as: (1) p53 modification using regulatory proteins that activate p53; (2) reactivating mutant p53; (3) adenovirus vector-mediated p53 transductions; and, (4) reactivating wt p53 by targeting the interaction between p53 and its negative regulator Mdm2 (Chen *et al.*, 2010). The rationale of investigating the impact of Nutlin-3 on NPC

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cells was based on the facts that: (1) Nutlin-3 has been explored as a specific p53 activator in wt-expressing cancer cells and is applicable in NPC which is rarely mutated; (2) Nutlin-3 chemo-protectively conserves normal cells; and, (3) Nutlin-3 integrates effectively with radiation, genotoxic or anti-mitotic agents in suppressing various tumours; but its effects on NPC have not been explored (Jiang *et al.*, 2007; Kojima *et al.*, 2005; Stuhmer *et al.*, 2005; Tovar *et al.*, 2006). Although NPC is an aggressive disease characterised by high levels of p53 (Sheu *et al.*, 2004) and several p53 gene mutations, it is considerably rare compared to other human cancers in general (Momand *et al.*, 1998; Lin *et al.*, 2014). Hence, antagonizing Mdm2 to restore p53 provides an alternative strategy to the current standard cancer therapy (Shangary and Wang, 2009). In this study, by exploiting the restoration of wt p53 with Nutlin-3 to maximise protection of normal cells and further enhance cisplatin action, the effectiveness of concurrent cisplatin and Nutlin-3 treatment against NPC cells are considered. In this interest, it is evident that Nutlin-3 provides sensitisation of tumour cells to cisplatin; hence reducing the dose required which could lead to lesser adverse effects while retaining stronger cytotoxic effect against NPC cells.

The disruption of p53-Mdm2 interaction to reactivate p53 pathway by Nutlin-3 is restricted to tumours which express wt p53 (Vassilev *et al.*, 2004), thereby a prior verification the p53 status of NPC and NPE cell lines was performed in the present study. A base change of G>C at codon 72 of the 4th exon of p53 gene, known as codon 72 polymorphism that encodes for variant amino acids: arginine (CGC) to proline (CCC) was previously reported (Matlashewski *et al.*, 1987). Here, the identical codon 72 polymorphism was detected in the p53 gene of NP69, NP460 and C666-1 cell lines. The polymorphism is common in wt p53 gene and has been detected in lung cancer (Fan *et al.*, 2000), teratoma (Udin *et al.*, 2008) and oesophageal squamous cell

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carcinoma (Yang *et al.*, 2008). As expected, the HK1 cell line has a homozygous point mutation C>G at codon 130 of the 5th exon of p53 gene, substituting leucine (CTC) with valine (GTC); similar to a previous report (Spruck *et al.*, 1992).

Initially, the response to cisplatin and Nutlin-3 treatment, singularly and in combination, was investigated in NPC C666-1 cells. The investigated cell lines were found to respond well to treatments; showing a greater loss of cell viability when treated with cisplatin alone as compared to only with Nutlin-3. Nutlin-3 had no effect on the loss of viability in NPE cells at the concentrations of >5 to 20 μM, consistent with recent studies that showed Nutlin-3 was not toxic to normal cells at 10–20 μM (Jiang *et al.*, 2007; Stuhmer *et al.*, 2005). The observation was more distinct on NPC cells where Nutlin-3 was found to reduce the viability of NPC cells dramatically at concentration as low as 5 μM. Taken together, the findings suggest that the effects of Nutlin-3 were more specific and selective towards cancer cells. In turn, at concentration of >20 μM, Nutlin-3 induced a dramatic growth inhibition effect on both NPC and NPE cells. Nutlin-3 completely impaired proliferation of NPE cell when its test concentration reached 40 μM. These findings suggest that Nutlin-3 inhibits cell proliferation in a dose-dependent manner, similar to a previous report which confirmed that the effect of Nutlin-3 on wt p53 osteosarcoma U-2 OS cells was concentration-dependent (Wang and Hu, 2012). Hence, this study demonstrated that further optimisation in clinical doses is important for enhancement of Nutlin-3 effects against cancer cells but with negligible effects on healthy cells at *in vitro* doses below 40 μM.

Cisplatin cytotoxicity was evaluated in NPE and NPC cell lines using various concentrations of cisplatin. The results showed that these cell lines had different reactions to cisplatin. Treatment with cisplatin at 10–20 μM, resulted in a dramatic

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effect on the reduction of viable cells, exerting greater cytotoxicity against NPE than to NPC cells. Treatment with ≥25 μM cisplatin almost completely inhibited the proliferation of NP460 cells. Conversely, NPC cells were more tolerant to cisplatin since ≥ 50% of cells remained viable following treatment with 30 μM cisplatin. The findings suggest that cisplatin inhibits proliferation of NPC and NPE cells in a dose-dependent manner. Furthermore, concurrent treatment of cisplatin and Nutlin-3 resulted in a stronger growth inhibitory effect on NPC cells when compared to single agent treatment. The combinations of 10 μM Nutlin-3 with 0–100 μM cisplatin; or 0–30 μM Nutlin-3 with 33.33 μM cisplatin showed significant inhibition on NPC cell proliferation when compared to the administration of cisplatin alone. The combinatorial treatments led to a dramatic reduction in the IC_{50} values of both drugs suggesting that when cisplatin given concurrently with Nutlin-3, it gains the largest anti-proliferative effect on NPC cell.

In addition, the effects of Nutlin-3 and/or cisplatin on tumourigenicity were determined in NPC C666-1 cells by assessing the abilities of colony formation in soft agar. The use of soft agar colony forming assays for the detection of *in vitro* tumourigenic potential of cancer cell transformation and anti-proliferation drug effects has become popular in oncologic research since 1977 (Salmon *et al.*, 1978). The clonal growth of single cell suspensions in semi-solid media was measured to detect anchorage-independent growth, the cell's ability to proliferate without attachment to a substratum, and its sensitivity to anti-proliferative agents.

The growth of the colonies was most easily achieved in the untreated-control, indicating that NPC C666-1 cells are capable of forming colonies in semi-solid media. Similarly, colonies appear to grow rapidly in the DMSO-treated control within the first

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week. The cumulative colony volume continued to increase and extend up to the second and third weeks, suggesting that 0.1% DMSO exerted no effect on the assay. Single treatment of cisplatin however, suppressed colony growth at day 7 and the effect was seen even up to day 21. Administration of Nutlin-3 alone was less effective in suppressing colony growth when compared to cisplatin. It was of interest to observe that the addition of 10 μM Nutlin-3 to cisplatin treatment further suppressed the tumourigenic ability of NPC cells. Having stronger suppression on colony growth further supports earlier observation that Nutlin-3 sensitizes NPC cells to the cytotoxic effect of cisplatin.

Several studies have reported that p53-Mdm2 interaction is significant for ubiquitination and proteasome degradation p53 in cancers retaining wt p53 (Fuchs *et al.*, 1998; Kubbutat *et al.*, 1997). As expected, Nutlin-3 activated p53 in parallel upregulation of p21 and Mdm2 in NPC C666-1 and colorectal carcinoma HCT116 cells expressing wt p53, but not NPC HK1 and breast cancer MDA-MB-231 cells lacking in wt p53. These observations suggest that Nutlin-3 activates the downstream p53 pathway in C666-1 cells; the effect of Nutlin-3 on apoptosis is highly correlated with the p53 status of NPC cells, which is in concordance with previous reports (Chang and Eastman, 2012; Kojima *et al.*, 2005; Van Maerken *et al.*, 2009). Furthermore, p21 protein was highly expressed in C666-1 and HCT116 cells when treated with Nutlin-3, suggesting that the expression of p21 is p53-dependent.

This study also further elucidated that wt p53 is a key factor in mediating apoptotic response to Nutlin-3. The function of p53 gene in C666-1 cells was blocked by lentiviral-based shRNA and the effect of p53-knocked down was investigated on Nutlin-3. As expected, the suppression of endogenous p53 inhibited the effects of

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Nutlin-3 on the p53, Mdm2 and p21 protein expressions in C666-1 cells. Furthermore, it impaired the effects of Nutlin-3 on cell survival in C666-1 cells, suggesting that Nutlin-3 activates the p53 pathway and exerts its cytotoxic effects on NPC cells in a p53-dependent manner. Therefore, the findings suggest that for such p53-based targeted therapy, determination of p53 status would be a prerequisite, since the effects of Nutlin-3 on NPC are p53-dependent. Taken together, the findings are in agreement with the idea that interference in p53-Mdm2 interaction is reserved for Nutlin-3; when the p53-Mdm2 interaction is disrupted by Nutlin-3, p53 is relieved from negatively regulating the autoregulatory feedback loop. Restoration of p53 subsequently led to accumulation of p53 and Mdm2 proteins in C666-1 cells, consistent with previous studies (Harris and Levine, 2005; Vassilev *et al.*, 2004).

On the other hand, the combinatorial treatment of NPC C666-1 cells with cisplatin and Nutlin-3 elucidated the largest inhibitory effect when compared to their IC_{50} values, and provided an early indication that the cells are probably moving towards apoptosis-related cell death. Apoptosis, a sequence of controlled self-destruction processes in programming cell death (Lockshin and Williams, 1964), was proposed by Kerr *et al.* as a morphologically noticeable mechanism for the removal of damaged cells (Kerr *et al.*, 1972). During early apoptosis, loss of plasma membrane (PM) integrity leads to translocation of phosphatidyl-serine (PS) to the outer leaflet of plasma membrane (Fadok *et al.*, 1992). The fluorochrome-labelled Annexin V has been developed for labelling PS exposure indicating the loss of PM asymmetry caused by apoptosis (Miyachi *et al.*, 2009).

In this study, apoptosis-related cell death was defined by detecting extrinsic appearance of PS in C666-1 cells using the Ca^{2+}-dependent phospholipid-binding FITC-

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labelled Annexin V. Nutlin-3 induction of apoptosis-related PS exposure in C666-1 cells was less effective compared to cisplatin. It was interesting to observe that combinatorial treatment of C666-1 cells with cisplatin and Nutlin-3 significantly induced apoptosis-related PS exposure in C666-1 cells, indicating that Nutlin-3 sensitises C666-1 cells to cisplatin-induced apoptosis. Similarly, the synergistic activities have been reported in neuroblastoma (Barbieri *et al.*, 2006), gastric cancer cells (Shinji *et al.*, 2011), ovarian cancer cells (Roser *et al.*, 2013) and testicular germ cell tumours (Bauer *et al.*, 2010).

Several literature reports suggested that wt p53 activates both the intrinsic and extrinsic apoptosis pathways. Decision on which pathway should be activated, apoptosis or DNA repair, is dependent on the strength of the cellular stress involved. Irreparable DNA damage in response to severe stress activates apoptosis pathway, whereas minor stress results in activation of survival signals for DNA repair (Vousden and Prives, 2009). In response to irreparable DNA damage, intrinsic pathway is the main apoptotic pathway activated by p53, where p53 induced the expression of PUMA and translocation of BAX to the mitochondria (Haupt *et al.*, 2003).

The findings of this study proved that Nutlin-3 can activate the intrinsic pathway via the induction of p53-mediated pro-apoptotic genes. The accumulation of BAX and PUMA proteins was detected in Nutlin-3-treated C666-1 cells, suggesting that the cells underwent apoptosis in response to irreparable DNA damage. This finding is consistent with a report that BAX and PUMA are apoptosis-related proteins (Miyachi *et al.*, 2009). Cisplatin alone had no effect on the expression of these proteins in C666-1 cells. The concurrent treatment of C666-1 cells with cisplatin and Nutlin-3 further enhanced the accumulation of BAX and PUMA proteins, indicating their larger effects on apoptosis.

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Furthermore, the detection of cleaved PARP level in the cells treated with the cisplatin and Nutlin-3 combination was consistent with apoptosis. Cleavage of structural protein between Asp214 and Gly215, such as DNA repair enzymes PARP-1, resulted in fragmentation of N-terminal (DNA binding domain 24 kDa) and C-terminal (catalytic domain 89 kDa), which hinders DNA repair enzymes from binding to the chromatin. Proteolysis of PARP prevents DNA repair and further facilitates apoptosis. Thus, the presence of 89 kDa PARP fragment is identified as a biomarker for apoptosis (Boulares *et al.*, 1999). Taken together, these findings are in agreement with the notion that p53 activation leads to apoptosis by activating p53 pro-apoptotic targets PUMA and BAX.

Collectively, Nutlin-3 is reported to affect cisplatin-induced apoptotic-cell death, triggering the expression of p53, and sensitising C666-1 cells to cisplatin-induced apoptosis by modulating the p53 pathway. Nevertheless, an issue that is deeply concerned to all is the emergence of p53 mutation in NPC. The mutant p53 and the reduction of p53 level have been detected in head and neck cancers due to loss of wt p53 properties (Gasco and Crook, 2003). NPC cells have increased levels of p53 (Sheu *et al.*, 2004); in fact p53 gene mutations are often found in relapse tumours, thereby becoming more resistant to chemotherapy (Gutekunst *et al.*, 2011; Houldsworth *et al.*, 1998). Moreover, Michaelis *et al.* (Michaelis *et al.*, 2011) and others (Kotchetkov *et al.*, 2003) have raised the serious concerns of how long-term treatment of cancer cells with Nutlin-3 could result in the emergence of Nutlin-3 resistant p53-mutated cells. However, the emergence of Nutlin-3-resistant NPC cells associated with p53 mutation has not been reported. The present study also investigated whether treatments with Nutlin-3 can lead to emergences of p53-mutated Nutlin-3-resistant NPC cells. Results obtained from this study showed that long-term exposure of NPC C666-1 cells to 10–40 μM Nutlin-3 resulted in reduced sensitivity to Nutlin-3 but not associated with the

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emergence of p53 gene alteration, suggesting that the acquisition of Nutlin-3 resistance in these cells could be due to other mechanisms. It is therefore suggested that optimisation of clinical doses and treatment duration is important to enhance the efficacy of Nutlin-3. However, such clinical implications have yet to be investigated.

Despite all the advances in cancer therapies, more drugs that reactivate the p53 pathway are entering clinical trials because the demand for alternative therapy to cure cancers keeps growing (Khoo *et al.*, 2014). Nonetheless, which tumour types have the probability of demonstrating effective clinical therapeutic index remain unpredictable. The preliminary trials on p53-activating therapies show that appropriate designing of clinical trials remains a crucial issue (Khoo *et al.*, 2014). Identifying tumours with low incidence p53 mutation and is highly responsive to p53-activating drugs, as well as determination of p53 status as a prerequisite for administering the drugs are vital in the design of such clinical trials. Moreover, p53 mutation's contribution to cancer development are expected to be associated with drug resistance, hence understanding the mechanism of drug resistance induction, particularly the inhibition of p53-induced apoptosis from extended treatments with p53-activating drugs, should be considered as well.

In the present study, Nutlin-3 is reportedly to be applicable in NPC as it demonstrated how specific p53 activation led to accumulation of p53 and integrates effectively with cisplatin therapy to elicit p53-dependent cell killing effect in NPC while sparing normal NPE cells. Notably, optimisation in clinical doses and treatment duration has been identified to be important in enhancing the efficacy of Nutlin-3 and the acquisition of Nutlin-3 resistance in NPC cells is not associated with the emergence of p53 gene alteration as well. To conclude, these findings have shed some illumination

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on understanding the use of Nutlin-3 as a p53-activating drug, which could be useful in developing and designing clinical trials for NPC treatment.

5.1 Recommendations and Future Studies

The present study provides indication that the establishment of p53-based targeted therapy offers greater efficacy of cisplatin-induced cell death in NPC, while maximizing protection against normal NPE cells. The strategy to conserve normal cells is not limited to targeting p53-Mdm2 interaction. Truly, there are possibilities for synergistic interaction between cisplatin and Nutlin-3. Based on the conclusive findings, few studies have been identified as future research for the development of p53-reactivating therapy in NPC.

Firstly, the synergistic effects via combination of low doses cisplatin and Nutlin-3 should be further examined and confirmed by evaluating the anticancer effects towards *in vivo* NPC in nude mice. The drug interaction study will be performed by determining multiple drug dose-effects based on Median Effect Methods described by T. C. Chou and P. Talalay (Chou, 2010; Chou and Talalay, 1984). The determination of dose-limiting toxicity seeks to facilitate dose reduction of each drug, hence refine the cytotoxicity of cisplatin against normal tissue. In addition, signaling pathways involved leading to the synergistic effects of the combination can be investigated *in vitro* and *in vivo.*

Secondly, further *in vivo* research needs to be performed to expedite the selectivity and specificity effects of Nutlin-3. The efficacy of Nutlin-3 in activating p53-dependent pathways, which selectively enhances cisplatin-induced apoptosis in NPC could be confirmed; and its chemo-protective properties, which provide effective protection

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towards normal tissues against the adverse-effects of cisplatin needs to be evaluated in *in vivo* NPC.

Lastly, the overexpression of p53, as well as the signaling pathways involved in the mechanism of Nutlin-3-resistant induction and emergence of p53 alteration in NPC could be evaluated; and it is also possible to examine their involvement and pathological significances in NPC in an attempt to understand the molecular basis of this disease. Similar findings are expected in *in vivo* NPC even though only one EBV-positive NPC cell line is tested *in vitro* in the present study. Ultimately, of course, the possibility of improved cell death warrants further investigation of Nutlin-3, which has been identified as future clinical trial for the development of p53-activating therapy in NPC.

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CHAPTER 6: CONCLUSION

The study has demonstrated that Nutlin-3 is an effective p53 activator in targeting the p53-Mdm2 interaction; it also activates the p53 pathway for sensitization of cisplatin-induced apoptosis in NPC cells in a p53-dependent manner. Nutlin-3 also suppresses anchorage-independent growth of NPC cells on soft agar when used in combination with cisplatin. Concurrently, Nutlin-3 strengthens the anti-proliferative activity of cisplatin via dose reduction, while retaining lesser cytotoxicity against non-malignant cells. Convincingly, the extended treatments of Nutlin-3 does not alter p53 gene in NPC cells. Nonetheless, the efficacy of the Nutlin-3 needs to be further optimised and supported by *in vivo* evaluation of NPC in nude mice; and if successful, followed by clinical studies as future research for the development of p53-activating therapies in NPC. In conclusion, the overall findings of the present study provide further insights for the potential use of Nutlin-3 as an NPC therapeutic agent.

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APPENDIX A

MEDIA AND REAGENT PREPARATION

0.25% Trypsin-EDTA

The ready-to-use 0.25% (w/v) trypsin was prepared in balanced salt buffer, with 1 mM EDTA and without calcium (Ca^{2+})/ magnesium (Mg^{2+}), purchased from Gibco Life Technologies, Carlsbad, CA, USA; and stored at 4 °C.

0.4% Trypan Blue

The ready-to-use 0.4% trypan blue was purchased from Gibco Life Technologies, Carlsbad, CA, USA and kept at room temperature.

0.5 M EDTA pH 8.0

Hundred ml of 0.5 M EDTA was prepared by dissolving 18.612 g EDTA (BDH, England) in 100 ml dH_2O. The pH was adjusted to 8.0 using 1.0 M sodium hydroxide (Sigma-Aldrich); and stored at room temperature.

10% Supplemented FCS Complete DMEM Medium

One hundred ml of 10% supplemented FCS DMEM medium were prepared using 90 ml of basic medium, supplemented with 10 ml heat-inactivated FCS, plus 0.5 ml of each streptomycin (2 000 µg/ml) and penicillin (2 000 U/ml), respectively. The medium was filter-sterilised using a 0.22 µm filter and stored at 4 °C.

10% Supplemented FCS Complete RPMI 1640 Medium

One hundred ml of 10% supplemented FCS RPMI 1640 medium were prepared using 90 ml of basic medium, supplemented with 10 ml heat-inactivated FCS, plus 0.5 ml of each streptomycin (2 000 µg/ml) and penicillin (2 000 U/ml), respectively. The medium was filter-sterilised using a 0.22 µm filter and stored at 4 °C.

15% Supplemented FCS Complete RPMI 1640 Medium

One hundred ml of 10% supplemented FCS RPMI 1640 medium were prepared using 85 ml of basic medium, supplemented with 15 ml heat-inactivated FCS, plus 0.5 ml of each streptomycin (2 000 µg/ml) and penicillin (2 000 U/ml), respectively. The medium was filter-sterilised using a 0.22 µm filter and stored at 4 °C.

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Antibiotic Penicillin and Streptomycin

Antibiotic stock solution of 100x penicillin (10 000 U/ml) and streptomycin (10 000 ug/ml) were obtained from Gibco Life Technologies, Carlsbad, CA, USA. Working concentrations of 2 000 U/ml penicillin and 2 000 µg/ml streptomycin were prepared by diluting stock solution with 1x PBS at a ratio of 1:5. Working solution was dispensed in convenient 10 ml aliquots and stored frozen at -20 °C. 500 µl working concentrations of each antibiotic were added to 100 ml of basal culture medium to obtain a final concentration of 10 U/ml penicillin and 10 µg/ml streptomycin.

Basal DMEM Medium

Basal DMEM medium was prepared by dissolving one sachet of DMEM powder with L-glutamine, 110 mg/L sodium pyruvate (Gibco Life Technologies, Carlsbad, CA, USA) and 3.7 g sodium bicarbonate in 1 L of distilled dH_20. The pH was adjusted to 7.2–7.4 using 5.0 N sodium hydroxide (NaOH) (Sigma-Aldrich). The medium was then filter-sterilised through a 0.2 µm filter and kept at 4 °C.

Basal RPMI 1640 Medium

Basal RPMI 1640 medium was prepared by dissolving one sachet of 10.4 g RPMI 1640 powder with L-glutamine (Gibco Life Technologies, Carlsbad, CA, USA), 2.0 g sodium bicarbonate (Sigma-Aldrich) and 5.94 g 4 (2-hydroxyethyl) piperazine-1-ethanesulfonic acids (HEPES) (Sigma-Aldrich) in 1 L of distilled dH_20. The pH was adjusted to 7.2–7.4 using 5.0 N sodium hydroxide (NaOH) (Sigma-Aldrich). The medium was then filter-sterilised through a 0.2 µm filter and kept at 4 °C.

Cells Cryopreservative Medium

Freezing medium consisting of heat-inactivated FCS, culture medium and pure culture grade DMSO in a ratio of 5:4:1 was prepared fresh for each cryopreservation step.

Cisplatin

The ready-to-use 1 mg/ml stock cisplatin [*cis*-$PtCl_2(NH_3)_2$] (Kemoplat, Fresenius Kabi India Pvt. Ltd.), chemotherapy drug solution was obtained from Hospital Kuala Lumpur, Malaysia. The stock solution was stored at room temperature for less than 6 months. The mixtures were well mixed by a vortex at high speed. A serial titration was performed to prepare working concentration with six folds higher than final concentration (0–40 µg/ml or 0–133.32 µM) used in the cytotoxicity MTS assay. To obtain the designed concentration, cisplatin was titrated with complete culture medium according to the formula described below;

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$$C_1V_1 = C_2V_2 \text{ .....Equation } 2$$

C_1 = original concentration of stock solution (1 mg/ml)
C_2 = final concentration of stock solution, after dilution
V_1 = volume of stock solution (1 mg/ml) about to be diluted
V_2 = final volume of stock solution (1 mg/ml), after dilution

Culture Medium for Human Keratinocytes NP460 hTERT

One hundred ml of NP460 culture medium (Gibco Life Technologies, Carlsbad, CA, USA) was prepared by adding 50 ml Define Keratinocyte-SFM basal medium to 100 µl Growth Factors Gibco, 50 ml EpiLife Defined Growth Supplement (EDGS) , 500 µl EDGS Growth Factors (Cascade Biology S-012-5), plus 1.0 ml of each streptomycin (1000 µg/ml) and penicillin (1000 U/ml), respectively. The medium was filter-sterilised using a 0.22 µm filter and stored at 4 °C.

Culture Medium for Human Keratinocytes NP69

The components of NP69 medium were obtained from Gibco Life Technologies, Carlsbad, CA, USA. One hundred ml of NP69 culture medium was prepared by adding 100 ml Keratinocyte-SFM basal medium to 250 µl Bovine Pituitary Extract (25 mg), 1.4 µl Recombinant Epidermal Growth Factor (rEGF) (2.5 µg), plus 0.5 ml of each streptomycin (2 000 µg/ml) and penicillin (2 000 U/ml), respectively. The medium was filter-sterilised using a 0.22 µm filter and stored at 4 °C.

Dimethyl Sulfoxide (DMSO)

Pure 99.5 percent culture grade DMSO was purchased from Sigma-Aldrich and kept at room temperature. Fresh 10% and 0.1% DMSO solution was prepared by mixing pure DMSO with culture media at a ratio of 1:10 and 1:1000 for cell storage and cytotoxicity MTS assay, respectively.

Fibronectin

The suspension of C666-1 cells was sub-cultured in an adherent flattened monolayer form in 10 cm-culture plate (Techno Plastic Products (TPP), AG, Switzerland). Fibronectin purchased from Sigma-Aldrich was used for C666-1 cell adhesion. Stock solution of 1 mg/ml fibronectin was prepared by dissolving 5 mg of powdered fibronectin in 5 ml of 1x PBS, aliquoted and stored at -20°C. The total 60 cm^2 surface area of the 10 cm-culture plate was pre-coated with 20 µg/ml fibronectin at 4°C for overnight prior to use.

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Foetal Calf Serum (FCS)

Foetal Calf Serum (FCS) purchased from Gibco Life Technologies, Carlsbad, CA, USA was kept frozen at -20°C. FCS was inactivated at 56°C for 45 min in a waterbath, followed by filtration through 0.22 µm and stored at -20°C. To prepare 10% and 15% FCS culture media, 10 ml and 15 ml of filtered FCS were added to 90 ml and 85 ml of basal culture media, respectively. Sterility test was performed at 37°C overnight incubation and media stock was stored at 4 °C for less than 3 months.

Nutlin-3

Nutlin-3 ($C_{30}H_{30}Cl_2N_4O_4$, 581.4896 g/mol), a cis-imidazoline analogue used in this study was obtained from Alexis, USA. Fresh stock solution of 10 mM Nutlin-3 was prepared by dissolving 0.006 g Nutlin-3 in 1:10 of culture grade 99.5% DMSO and culture medium. The mixtures were well mixed by high speed vortex. Working solution of 1 mM Nutlin-3 was then prepared by adding 100 µl of stock solution to 900 µl of culture medium. A panel of treatment concentration (0–80 µM) was obtained by titrating the working solution with complete culture medium according to Equation *2.* The final concentration of DMSO in the working Nutlin-3 solution involved in this study was adjusted to $\leq 0.1\%$ with complete culture medium.

Phosphate Buffered Saline (PBS) pH7.2

Dulbecco's formula (modified) without magnesium and calcium phosphate buffered saline tablet was purchased from Takara Bio Inc. The 1x PBS solution was prepared by dissolving a tablet in 100 ml dH_20. The buffer was then sterilised using 0.22 µm filter (Merck, Millipore, US) and autoclaved before storage at 4°C.

Tris-Borate-EDTA (TBE) Buffer

One litre of 10x TBE was prepared by adding 10.8 g Tris base (Acros Organics, USA) and 5.5 g boric acid (BDH, England) to 4.0 ml of 0.5 M EDTA at pH 8.0 (BDH, England).

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APPENDIX B

ISI-CITED PUBLICATION

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Nutlin-3 sensitizes nasopharyngeal carcinoma cells to cisplatin-induced cytotoxicity

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Abstract. The small-molecule inhibitor of p53-Mdm2 interaction, Nutlin-3, is known to be effective against cancers expressing wild-type (wt) p53. p53 mutations are rare in nasopharyngeal carcinoma (NPC), hence targeting disruption of p53-Mdm2 interaction to reactivate p53 may offer a promising therapeutic strategy for NPC. In the present study, the effects of Nutlin-3 alone or in combination with cisplatin, a standard chemotherapeutic agent, were tested on C666-1 cells, an Epstein-Barr virus (EBV)-positive NPC cell line bearing wt p53. Treatment with Nutlin-3 activated the p53 pathway and sensitized NPC cells to the cytotoxic effects of cisplatin. The combined treatment also markedly suppressed soft agar colony growth formation and increased apoptosis of NPC cells. The effect of Nutlin-3 on NPC cells was inhibited by knockdown of p53, suggesting that its effect was p53-dependent. Extended treatment with increasing concentrations of Nutlin-3 did not result in emergence of p53 mutations in the C666-1 cells. Collectively, the present study revealed supportive evidence of the effectiveness of combining cisplatin and Nutlin-3 as a potential therapy against NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a common epithelial squamous-cell head and neck carcinoma, which originates from the nasopharyngeal mucosa and is associated with Epstein-Barr virus (EBV) infection (1). This type of cancer shows a distinct racial and geographical distribution and is prevalent among Southern Chinese, as well as natives in Southeast Asia including Malaysia (2,3). In the early stages, NPC may be asymptomatic or present with apparently trivial symptoms and is therefore likely to be ignored resulting in late diagnosis (4,5).

NPC is usually treated with radiotherapy and/or chemotherapy (cisplatin and 5-fluorouracil) (6). Concurrent chemo-radiotherapy is the main modality of treatment for advanced-stage NPC (7). However, advanced-stage NPC is associated with poor prognosis and therapeutic failure (7). Recurrence, distant metastases, treatment resistance and adverse effects of treatment remain the major challenges in the treatment of NPC (8-10).

p53, a tumor suppressor gene, is a transcription factor which controls genes involved in DNA repair, cell cycle arrest and apoptosis (11). Murine double minute (Mdm2) is an important negative regulator of p53 (12). It is an E3 ubiquitin ligase which promotes degradation of p53 via the ubiquitin-proteosome pathway (13,14) and functions in an autoregulatory feedback loop with p53. Inhibition of the p53-Mdm2 interaction prevents this degradation, thus resulting in increased p53 levels.

Nutlins are *cis*-imidazoline analogs (14) which compete with Mdm2 for binding to p53. Nutlin-3 has been reported to be effective in killing cancer cells retaining functional wt p53 (15). Nutlin-3 exerts anticancer effects on acute myeloid (16) and chronic lymphocytic leukemia (17), multiple myeloma (18), Kaposi sarcoma (19), liposarcoma (20), rhabdomyosarcoma (21), Ewing's sarcoma (22), colon (23) and testicular cancer (24), osteosarcoma and other types of cancer (25). Nutlin-3 suppressed not only tumor growth, but also distant metastasis in a xenograft model of wt p53 neuroblastoma (26). Moreover, Nutlin-3 selectively enhanced apoptosis in wt p53 cancer cells by activating the p53 pathway (16,26). Currently, drugs which reactivate the p53 pathway are undergoing clinical trials (27). Although Nutlin-3 has been reported to be effective against a wide variety of tumors bearing wt p53, the effects of Nutlin-3 on NPC cells have yet to be reported.

p53 mutations have been reported to be rare in NPC (28,29), even in recurrent radioresistant NPC (30), thus

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Key words: apoptosis, C666-1 cells, drug resistance, p53 knockdown, p53-Mdm2 targeted therapy, synergism, Nutlin-3

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making this cancer a potential candidate for treatment with p53-Mdm2 inhibitors, such as Nutlin-3. However, increased staining of p53 protein by immunohistochemistry has been reported, suggesting the deregulation of the p53 pathway in NPC (29,31,32). In addition, latent genes of EBV, such as LMP1 which is known to be expressed in NPC, has been reported to inhibit the p53 pathway (33). Interestingly, it has been suggested that induced overexpression of p53 using an adenoviral vector was found to be effective against NPC cells (34,35), indicating that further elevation of the p53 levels using the p53 activator, Nutlin-3, may be effective to further improve NPC treatment.

In the present study, we sought to investigate the effects of Nutlin-3 alone or in combination with cisplatin on an EBV-positive, wt p53, NPC cell line, C666-1. We also tested whether extended treatment with Nutlin-3 would result in the emergence of p53 mutations in NPC cells. The findings of the present study provide further insights for the potential use of Nutlin-3 as an NPC therapeutic agent.

Materials and methods

In vitro cell culture. C666-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 15% heat-inactivated fetal calf serum (FCS) (Gibco Life Technologies). EBV-negative NPC HK1 cells were cultured in 10% FCS RPMI-1640 medium. HCT116, a colorectal carcinoma cell line, and MDA-MB-231, a metastatic breast adenocarcinoma cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies) supplemented with 10% heat-inactivated FCS. Nasopharyngeal epithelial (NPE) cell line, NP69, was cultured in keratinocyte serum-free medium (K-SFM) (Gibco Life Technologies) supplemented with bovine pituitary extract and 0.16 ng/ml recombinant epidermal growth factor (Gibco Life Technologies), while NPE NP460 cells were maintained in a 1:1 ratio of defined K-SFM medium supplemented with growth factor (Gibco Life Technologies) and EpiLife® Defined Growth Supplement (Cascade Biologics, Life Technologies, Carlsbad, CA, USA). All of the cell lines were maintained in an exponential growth phase in the presence of 10 U/ml of penicillin (Gibco Life Technologies) and 10 μg/ml streptomycin (Gibco Life Technologies) at 37°C in a 5% CO_2 humidified atmosphere. Mycoplasma-free status of the cells was verified monthly with the e-myco™ Mycoplasma PCR Detection kit (Intron Biotechnology, Inc., Seongnam, Korea).

PCR and DNA sequencing. Genomic DNA was extracted from cultured cells using QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. p53 gene amplification spanning from exons 2 to 11 was amplified in separate PCR reactions using specific designed oligonucleotide forward (F) and reverse (R) primers synthesized by First BASE Laboratories, Malaysia. Primer sequences used for PCR were: exon 2 (F), 5'-AGC TGT CTC AGA CAC TGG CA-3' and (R), 5'-GAG CAG AAA GTC AGT CCC ATG-3'; exon 3+4 (F), 5'-AGA CCT ATG GAA ACT GTG AGT GGA-3' and (R), 5'-GAA GCC TAA GGG TGA AGA GGA-3'; exon 5 (F), 5'-CGG AAT TCT TAT CTG TTC ACT TGT GCC C-3' and (R), 5'-CGG GAT CCA CCC TGG GCA ACC AGC CCT G-3'; exon 6 (F), 5'-CGG AAT TCG GTC CCC AGG CCT CTG ATT CCT -3' and (R), 5'-CGG GAT CCA CCC GGA GGG CCA CTG ACA AC-3'; exon 7 (F), 5'-CTG CTT GCC ACA GGT CTC-3' and (R), 5'-TGG ATG GGT AGT AGT ATG GAA G-3'; exon 8 (F), 5'-CGG AAT TCT TGG GAG TAG ATG GAG CCT-3' and (R), 5'-CGG GAT CCC TCC TCC ACC GCT TCT TGT CCT-3'; exon 9 (F), 5'-AGC AAG CAG GAC AAG AAG CG-3' and (R), 5'-CCA GGA GCC ATT GTC TTT GA-3'; exon 10 (F), 5'-CTC AGG TAC TGT GTA TAT ACT TAC-3' and (R), 5'-ATA CTA CGT GGA GGC AAG AAT-3'; exon 11 (F), 5'-TCC CGT TGT CCC AGC GTT-3' and (R), 5'-TAA CCC TTA ACT GCA AGA ACAT-3'. The quality and purity of the resulting PCR products were evaluated on an ethidium bromide stained-agarose gel (2%). The products were further purified using Qiagen QIA quick PCR Purification kit followed by Sanger sequencing. The data were aligned and compared against the published human p53 sequences in NCBI GenBank (X54156). The nucleotide variants were analyzed using Mutation Surveyor V4.0.7 (SoftGenetics, LLC., State College, PA, USA).

Western blot analysis. Cells were lysed using cold lysis buffer containing 2 mM DL-dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride (Bio-Rad, Hercules, CA, USA) and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Total protein lysate (10 μg) was separated on 10-15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by immunoblotting with monoclonal antibodies for p53 (Promega Corporation, Madison, WI, USA), Mdm2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p21Waf1/Cip1, PUMA, BAX, Bcl2 and PARP (Cell Signaling Technology, Inc., Beverly, MA, USA). β-actin (Santa Cruz Biotechnology) was used as a loading control and secondary antibody reactions were performed with anti-mouse or anti-rabbit (Promega Corporation) horseradish peroxidase-conjugated IgG. Chemiluminescent imaging was conducted using ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Buckinghamshire, UK). Densitometric analysis was performed on an Alpha Imager HP System with AlphaView software (Alpha Innotech Corporation, San Leandro, CA, USA).

MTS cell viability assay. Ready-to-use 1 mg/ml stock cisplatin [*cis*-$PtCl_2(NH_3)_2$] (Kemoplat, Fresenius Kabi, Maharashtra, India) and Nutlin-3 (Alexis Biochemicals, San Diego, CA, USA) were utilized for the *in vitro* treatment. Briefly, cells were seeded at densities of $1.5x10^4$ cells/ml for NP69 and NP460; and $3x10^4$ cells/ml for C666-1 cells in 96-well plates (TPP Techno Plastic Products AG, Trasadingen Switzerland). The cells were cultured for 24 h before treatment. The dose-response curves and half maximal inhibitory concentration (IC_{50}) values were determined by 96® AQueous One Solution Cell Proliferation MTS solution (Promega Corporation) followed by measurement using an EnVision multilabel plate reader (PerkinElmer, Waltham, MA, USA).

Soft agar colony formation assay. The C666-1 cells were seeded at a density of $5x10^4$ cells/ml in 96-well plates (TPP), followed by cisplatin treatment with or without Nutlin-3.

The cells were then plated into a two-layer soft agar made from DNA grade Seakem agarose (Lonza, Rockland, ME, USA) culture system (comprised of a layer of 0.3% agarose in complete media; and with 0.6% agar as a base layer) in 6-well plates (TPP). Anchorage-independent growth was measured by counting the numbers of viable colonies using an Olympus stereomicroscope model SZX7 (Olympus, Tokyo, Japan). The colonies were scored by using Image Pro Plus AMS version 6.3 (Media Cybernetics, Inc. Rockville, MD, USA). Colonies with a minimum diameter of 60 μm, area 2,800 μm^2 and roundness score ranging from 0.25 to 0.50 (roundness $=4A/\pi D^2$; A is the area; D is the maximum diameter; with 1.0 indicating a perfect circle) were counted in order to exclude abortive colonies.

p53 knockdown with small-hairpin RNA (shRNA). p53 knockdown was performed using four lentiviral-based shRNA constructs (Sigma-Aldrich, St. Louis, MO, USA). The shRNA p53-target sequences were as follows: p53si-2 (D3), 5'-CAC CATCCACTACAACTACAT-3'; p53si-3 (C12), 5'-CGGCGC ACAGAGGAAGAGAAT-3'; p53si-4 (E1), 5'-GAGGGATGT TTGGGAGATGTA-3'; p53si-5 (E2), 5'-GTCCAGATGAAG CTCCCAGAA-3' and non-specific (NS), 5'-CAACAAGAT GAAGAGCACCAA-3'. Lentiviral stocks were generated by co-transfecting the HEK-293T cells (ATCC® CRL-3216™; American Type Culture Collection, Manassas, VA, USA) with the plasmid vector, the psPAX2 packaging plasmids (Addgene plasmid 12260) and pMD2G envelope plasmid (Addgene plasmid 12259) (Addgene, Inc., Cambridge, MA, USA) using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The knockdown was verified by western blot analysis.

High content analysis of apoptosis. Briefly, C666-1 cells were seeded at a density of $3x10^4$ cells/ml in View Plate-96 Black 96-well plates (PerkinElmer) and were allowed to grow for 24 h prior to cisplatin and/or Nutlin-3 treatments for 48 and 72 h. The cells were cultured with 0.1% DMSO (Sigma-Aldrich) or basal media, which served as vehicle controls for Nutlin-3 and cisplatin treatments, respectively. The cells were stained with AnnexinV-FITC, propidium iodide (PI) and Hoechst 33342 (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Well to well imaging with three filter channels (DAPI, FITC and TRITC) was performed using a Metamorph screening acquisition module, on a Nikon Ti-ECLIPSE inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan), at a magnification of x20. Nine fields were imaged and scored for each well using Metamorph software version 7.7.0.0 (Molecular Devices, Downingtown, PA, USA). The percentages of apoptotic cells were calculated from triplicate wells.

Establishment of Nutlin-3-resistant NPC C666-1 cells. Nutlin-3-resistant cells were generated by propagation of parental C666-1 cells in stepwise ascending concentrations (10, 20 and 40 μM) of Nutlin-3 for a varying total number of passages (7-36) over a period of up to six months. Cell viability of the Nutlin-3-resistant sublines relative to the control parental C666-1 cells was determined by MTS viability assay after a 72-h treatment with Nutlin-3. The resistance index (R) (R= IC_{50} resistant cells/IC_{50} sensitive cells) was calculated to determine the degree of acquired resistance to its relative control parental C666-1 cells.

Statistical analysis. Data were analyzed by Microsoft Excel and/or GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was measured using the Student's paired t-test and P-values <0.05 were considered to be statistically significant.

Results

p53 mutation status of NPC and NPE cells. In the present study, p53 sequences in NPC (C666-1; HK1) and NPE (NP69; NP460) cell lines were first examined by sequencing exons 2 through 11 of the p53 gene. The p53 sequences in C666-1 cells had a single G>C base substitution detected at position 12139 at codon 72 of exon 4, while no alteration was detected in other exons (data not shown). An identical alteration was also detected in the NP69 and NP460 cell lines. However, a homozygous C>G base substitution at codon 130 of exon 5 was detected in the HK1 cells. Overall, these results suggest that the p53 mutation was only detected in the HK1 cells, while C666-1, NP69 and NP460 cells retained the wt p53 status while expressing a polymorphism at codon 72, common in the wt p53 gene.

Effects of single drug treatments of cisplatin and Nutlin-3 on NPC and NPE cells. The effects of single drug treatment, cisplatin (Fig. 1A) or Nutlin-3 (Fig. 1B), on the growth and viability of the NPC C666-1 and NPE cell lines were evaluated. The results indicated that both NPC and NPE cells were sensitive to cisplatin and showed clear dose dependence, whereas the NPE cells were less sensitive than the NPC cells to Nutlin-3. The IC_{50} values showed that cisplatin was more cytotoxic against the NPE cell lines compared to the NPC cells (Table I). In contrast, Nutlin-3 showed stronger cytotoxic effects against the NPC cells compared to the effects in the NPE cells, suggesting that Nutlin-3 may be more selective towards cancer cells.

Combination treatment of cisplatin and Nutlin-3 on NPC cells. The effects of Nutlin-3 in combination with cisplatin on the C666-1 cells were investigated (Fig. 2). Cisplatin alone inhibited the growth of the C666-1 cells in a dose-dependent manner with an IC_{50} value of 26.55±6.15 μM. When C666-1 cells were treated with a combination of cisplatin (0-100 μM) and a fixed concentration of 10 μM Nutlin-3, the IC_{50} value was markedly reduced to 3.67±0.88 μM (Fig. 2A). Similarly, when the cells were treated with a combination of Nutlin-3 (0-30 μM) and a fixed concentration of 33.33 μM cisplatin, the IC_{50} value of Nutlin-3 was markedly decreased from 10.23±0.87 μM to 0.86±0.13 μM (Fig. 2B), suggesting that the cisplatin combined with Nutlin-3 was more effective than each agent when used separately. These findings provide an early indication that Nutlin-3 sensitizes C666-1 cells to the cytotoxic effects of cisplatin.

Effects of Nutlin-3 on the tumorigenicity of NPC cells in soft agar. The effects of Nutlin-3 in combination with cisplatin on the tumorigenicity of C666-1 cells were assessed by determining

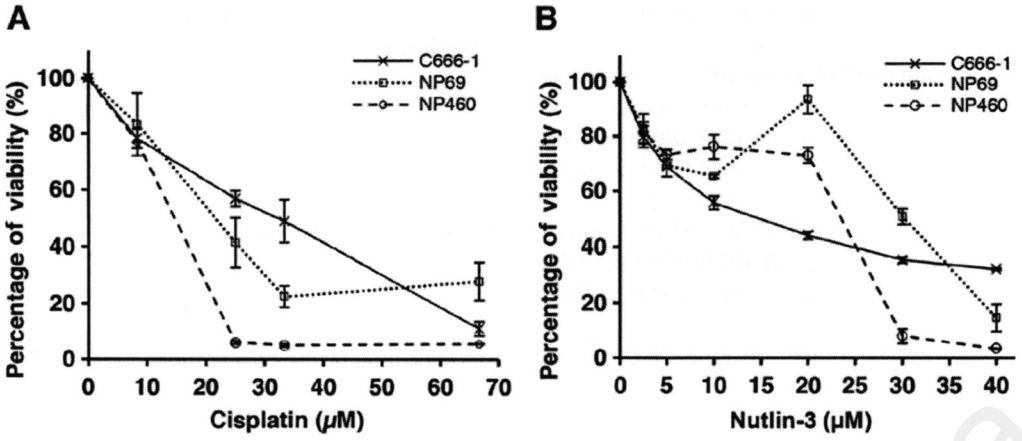

Figure 1. Growth-inhibitory effects of cisplatin and Nutlin-3 on NPC (C666-1) and NPE (NP69, NP460) cells. Cells were treated with increasing concentrations of (A) cisplatin (0-66.64 μM) or (B) Nutlin-3 (0-40 μM) for 72 h. Cell viability was determined by MTS viability assay. The average percentage of cell viability relative to the untreated and 0.1% DMSO-treated controls are shown.

Table I. Sensitivity of the NPC and NPE cell lines to cisplatin and Nutlin-3 as indicated by IC_{50} ± SD values.

	IC_{50} values (μM)	
Cell lines	Cisplatin	Nutlin-3
NP69	19.31±4.75	31.69±2.54
NP460	14.18±2.97	22.85±1.18
C666-1	32.07±4.18	19.95±8.93

Representative IC_{50} ± SD values are the mean of three independent experiments with nine data points. IC_{50}, half maximal (50%) inhibitory concentration; SD, standard deviation.

the efficiency of anchorage-independent colony formation using soft agar colony formation assay. Cisplatin (33.33 μM) and Nutlin-3 (10 μM) significantly reduced the number of colonies formed when administered alone as compared to the untreated and DMSO-treated controls. When the two treatments were combined, the numbers of colonies formed were markedly reduced (Fig. 2C) and the sizes of colonies were also significantly smaller (Fig. 2D). This further supports the earlier observation that Nutlin-3 sensitizes C666-1 cells to the cytotoxic effect of cisplatin and suppresses colony formation.

Nutlin-3 activates the p53 pathway in wt p53 NPC cells. The effects of Nutlin-3 on the p53 pathway were investigated on NPC C666-1 and HK1 cells harboring wt and mutant p53, respectively. Investigation was also performed in parallel with colorectal (HCT116) and breast (MDA-MB-231) cancer cells harboring wt and mutant p53, respectively. The expression levels of cellular proteins p53, p21Waf1/Cip1 and Mdm2 are shown in Fig. 3A. Upon treatment of C666-1 or HCT116 cells with Nutlin-3, p53 was activated and this significantly induced the expression of p21 protein and to a lesser extent, Mdm2 protein. The effects of Nutlin-3 were stronger in cells bearing wt p53 compared to cells with mutated p53 (HK1 and MDA-MB-231 cells). These findings suggest that Nutlin-3 activates the p53 pathway and induces upregulation of p53, p21 and Mdm2 in cells bearing wt p53.

Nutlin-3 activates the p53 pathway and exerts its cytotoxic effects on NPC cells in a p53-dependent manner. To verify that the cytotoxic effects of Nutlin-3 are mediated through the p53 pathway, we knocked down the p53 gene in the C666-1 cells using four different lentiviral-based shRNA constructs (p53si-E1, -E2, -C12 or -D3). The efficiency of knockdown was verified by examining the p53 protein expression level as shown in Fig. 3B; Vector-pLKO and NS were used as controls. Cells transduced with p53si-E2 and p53si-D3 constructs had 70 ($P<0.05$) and 90% ($P<0.005$) knockdown of p53 protein expression, respectively; whereas, the control Vector-pLKO and NS had unaltered p53 protein expression. Next, the effects of Nutlin-3 on the expression of p53-related proteins following the knockdown of p53 protein in the lenti-shΔp53-transduced cells were studied. When the parental C666-1 cells were exposed to 10 μM Nutlin-3, increased expression levels of p53, p21 and Mdm2 were observed in comparison to the untreated-parental C666-1 cells and control Vector-pLKO and NS (Fig. 3C). However, these effects were markedly inhibited in the p53-knockdown C666-1 cells transduced with the p53si-D3 or p53si-E2 construct. The percentages of viability relative to control Vector-pLKO and NS following treatment with Nutlin-3 are shown in Fig. 3D. Cells with p53 knockdown were found to be less sensitive to Nutlin-3 in comparison to the controls. Collectively, these findings suggest that the effect of Nutlin-3 on C666-1 cell viability is p53-dependent.

Nutlin-3 sensitizes NPC cells to cisplatin-induced apoptosis. As shown in Fig. 2, the combination of cisplatin and Nutlin-3 effectively impaired cell viability and markedly suppressed the tumorigenicity of C666-1 cells. We used high content imaging of Annexin V/PI-stained cells to determine the effects of the drug combination on induction of apoptosis (Fig. 4A and B). Treatment of C666-1 cells with cisplatin resulted in apoptosis. Apoptosis increased significantly in the cells treated with both cisplatin and Nutlin-3. These observations indicated that Nutlin-3 sensitized C666-1 cells to cisplatin and enhanced

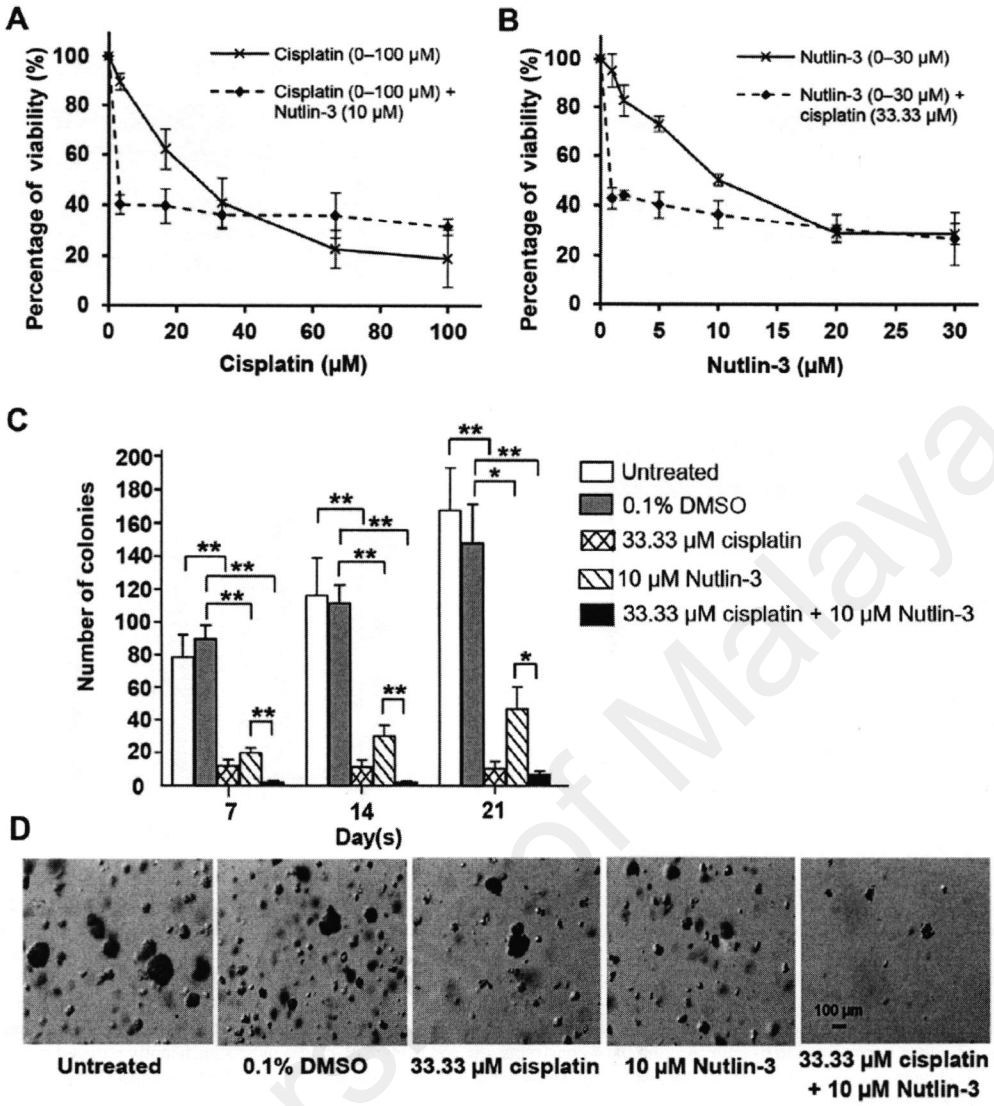

Figure 2. Nutlin-3 sensitizes C666-1 cells to the cytotoxic effect of cisplatin. The cell viability in response to (A) cisplatin alone (0-100 μM) and in combination with 10 μM Nutlin-3, and (B) Nutlin-3 alone (0-30 μM) and in combination with 33.33 μM cisplatin were assayed by MTS viability assay following 48 h of treatment. The graphs shown are representative average percentages of cell viability relative to the untreated and 0.1% DMSO-treated controls of three independent experiments with nine data points. (C) Effect of Nutlin-3 alone and in combination with cisplatin on colony formation of C666-1 cells. The plot shown is representative of two independent experiments carried out in triplicate. The untreated and 0.1% DMSO-treated cells were included as controls for cisplatin and Nutlin-3 treatments, respectively. Statistical analysis using the paired Student's t-test was performed to compare the difference in number of colonies scored on day 7, 14 and 21. *P<0.05 and **P<0.005 compared to the control. (D) Morphological appearances of C666-1 cell colonies in soft agar cultures. The image of the colonies was captured on day 21 using an Olympus stereomicroscope at a magnification of x2.5 (scale bar, 100 μm).

apoptotic cell death. Next, we investigated whether Nutlin-3 activates other p53-mediated pro-apoptotic genes (Fig. 4C). Treatment with Nutlin-3 alone significantly upregulated BAX and PUMA protein expression. Similarly, the expression levels of p53, BAX and PUMA were further enhanced by the combination of cisplatin and Nutlin-3. Furthermore, the cleaved PARP level detected in the cells treated with the combination drugs was consistent with apoptosis. Collectively, these results suggest that Nutlin-3 sensitized the C666-1 cells to cisplatin-induced apoptosis by modulating pro-apoptotic targets PUMA and BAX.

Extended treatment with Nutlin-3 results in reduced sensitivity without emergence of p53 mutation. p53 mutations are known to result in resistance to Nutlin-3 (36). To investigate whether Nutlin-3 induces the emergence of p53 mutant cells, C666-1 cells were treated with various concentrations of Nutlin-3 for extended periods (Fig. 5A). The Nutlin-3-treated NPC sublines showed a doubling in the IC_{50} value to Nutlin-3 when compared to the parental C666-1 cells, suggesting that the C666-1 cells were developing relative resistance to Nutlin-3 (Fig. 5B). Next, the Nutlin-3-resistant sublines were investigated for p53 status. The p53 gene sequence (spanning exon 2 to 11) of the investigated sublines was found to be wt compared to the parental C666-1 cells. These results indicate that, while extended treatment of NPC cells with Nutlin-3 resulted in decreased sensitivity to Nutlin-3, it was not associated with emergence of p53 mutations at the investigated doses and durations.

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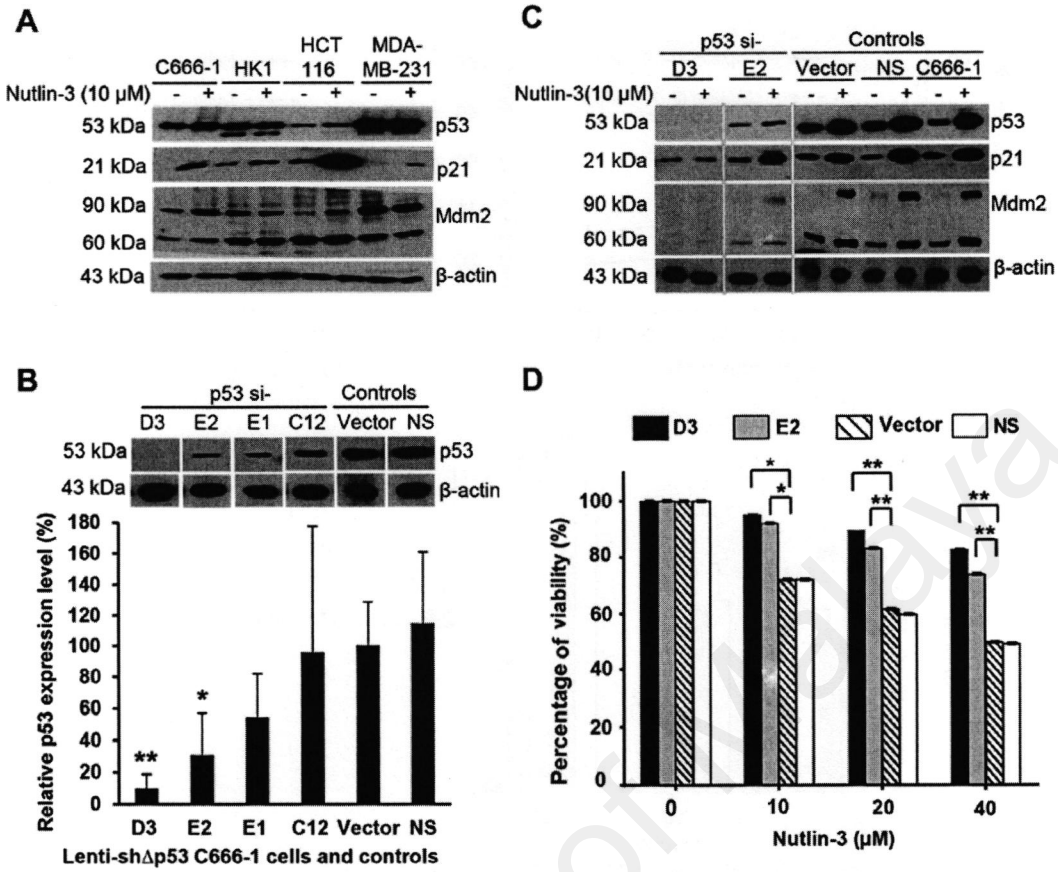


Figure 3. Nutlin-3 activates the p53 pathway, upregulating p53, p21 and Mdm2 in C666-1 cells in a p53-dependent manner. (A) The expression of p53-related proteins in cancer cells bearing wt p53 (C666-1 and HCT116) and mutant p53 (HK1 and MDA-MB-231) after being treated with 10 μM Nutlin-3 (+) or 0.1% DMSO (-) for 24 h are shown. β-actin is included as loading control. Nutlin-3 induced the upregulation of p53, p21 and Mdm2 proteins in the C666-1 and HCT116 cells bearing wt p53. (B) Establishment of p53 knockdown. p53-knockdown C666-1 cell sublines (lenti-shΔp53 C666-1) were generated using the lentiviral-based shRNA constructs p53si-E1, -E2, -C12 or -D3 in parallel with the control vector and NS. The efficiency of knockdown was verified at 96 h post transduction. The plot shown is representative of three independent experiments. The mean percentage of the relative p53 expression level was obtained by densitometric analysis when normalized to β-actin. Among the 4 constructs, the p53 protein level was most significantly reduced by p53si-D3 or p53si-E2. (C) The effects of Nutlin-3 on the expression of p53, p21 and Mdm2 proteins in the lenti-shΔp53 C666-1 cells. The lenti-shΔp53 C666-1 and control cells were treated with 10 μM Nutlin-3 for 24 h prior to analysis. The attenuation of the activation of the p53 pathway was evident in cells transduced with p53si-D3 or p53si-E2 compared to the controls. (D) Growth inhibitory effect of Nutlin-3 on lenti-shΔp53 C666-1 cells. Cell viability responses to 0-40 μM Nutlin-3 after a 72-h treatment was quantified by MTS viability assay. The average percentage of cell viability relative to the 0.1% DMSO-treated vehicle control is shown for the lenti-shΔp53 C666-1 cells and controls. The plot shown is representative of mean values of three independent experiments with nine data points. *P<0.05 and **P<0.005 compared to the vector. Vector, vector-pLKO; NS, non-specific.

Discussion

Targeting p53-Mdm2 interaction to reactivate p53 tumor-suppressing function is a promising cancer therapeutic strategy for tumors retaining wt p53 (37). The disruption of p53-Mdm2 interaction to reactivate the p53 pathway by small-molecule inhibitor Nutlin-3 is restricted to tumors with wt p53 (14). Hence, in the present study, a prior verification of the p53 status of our NPC and NPE cell lines was performed. A base change of G>C at codon 72 of the 4th exon of the p53 gene, known as codon 72 polymorphism which encodes for variant amino acids: arginine (CGC) or proline (CCC) was previously reported (38). In the present study, we found the identical codon 72 polymorphism in the p53 gene of our NP69, NP460 and C666-1 cell lines. This polymorphism is common in the wt p53 gene and has been detected in lung cancer (39), teratoma (40) and esophageal squamous cell carcinoma (41). As expected, our HK1 cell line had a homozygous point mutation C>G at codon 130 of the 5th exon of p53 gene, substituting leucine (CTC) with valine (GTC), similar to a previous report (42).

The present study demonstrated that Nutlin-3 sensitized NPC cells to cisplatin-induced cytotoxicity in a p53-dependent manner. Nutlin-3 reduced the viability and colony formation of wt p53 NPC cells. Furthermore, Nutlin-3 significantly reduced the viability of NPC, but not NPE cells at a concentration of 10-20 μM, consistent with recent studies that showed that Nutlin-3 was not toxic to normal cells at the concentrations of 10-20 μM (18,43).

Nutlin-3 activated p53 and upregulated p21 and Mdm2 in the C666-1 and HCT116 cancer cells retaining wt p53, but not in the HK1 and MDA-MB-231 cancer cells lacking wt p53. These findings are in concordance with previous reports that Nutlin-3 induced apoptosis in p53 wt, but not p53 mutant cancer cells (14,16,26,44). However, Nutlin-3 has also been reported to act via p53-independent pathways (45).

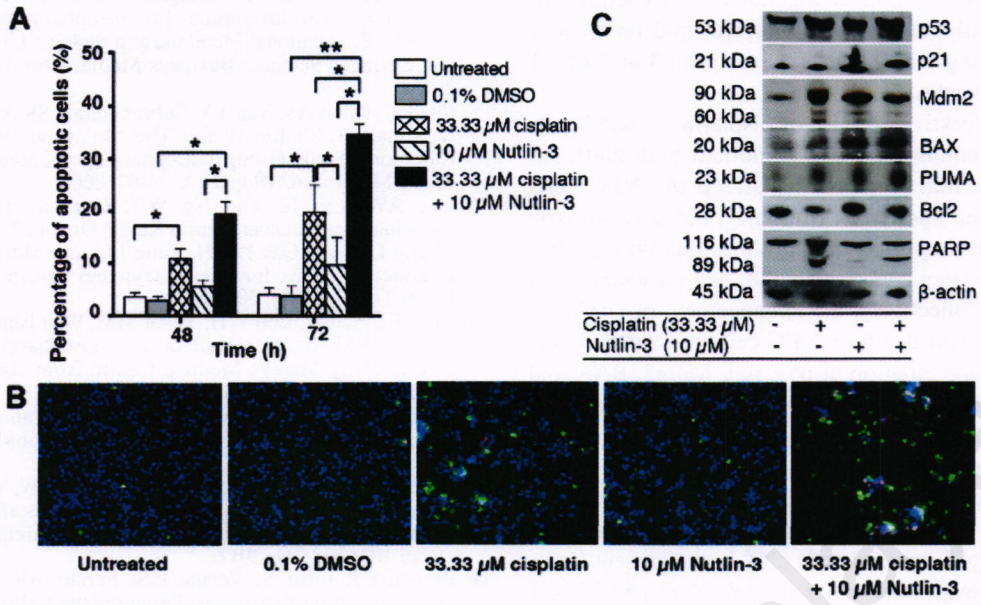
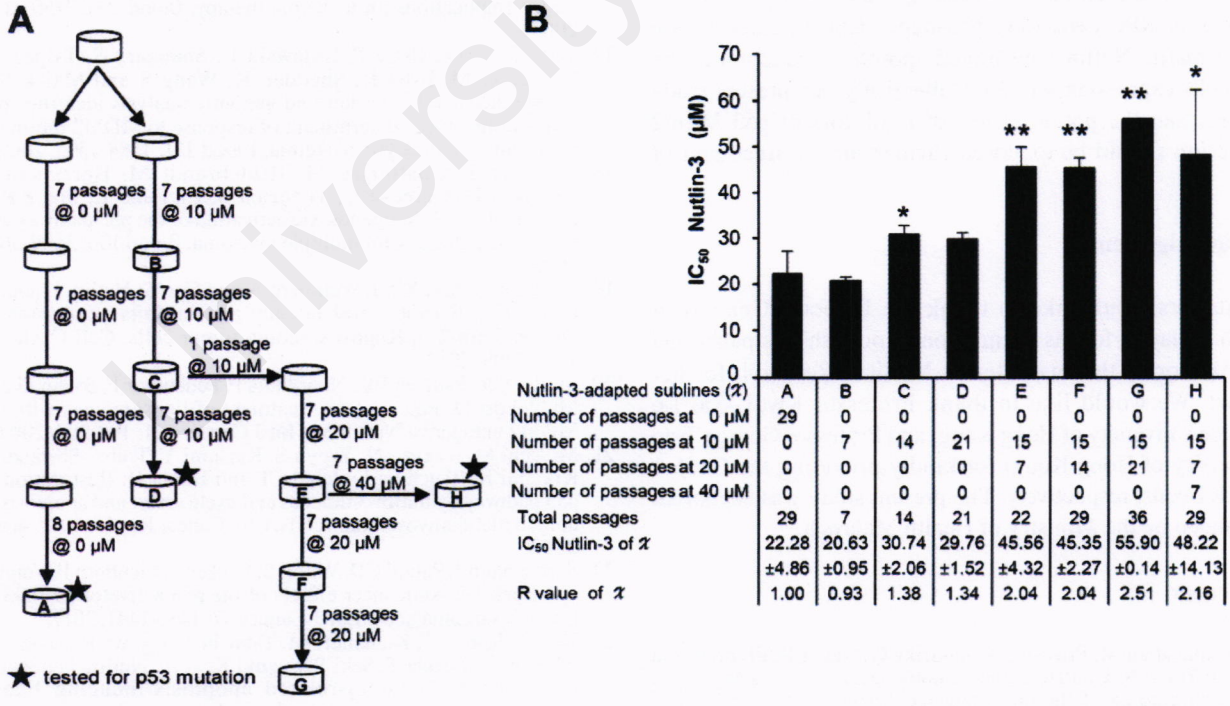


Figure 4. Nutlin-3 sensitizes C666-1 cells to cisplatin-induced apoptosis. (A) High content analysis of Annexin V-FITC/PI-stained cells to measure apoptosis induced by cisplatin and/or Nutlin-3 in C666-1 cells. The cells were treated with 33.33 μM cisplatin and/or 10 μM Nutlin-3 for 48 and 72 h prior to analysis. The untreated and 0.1% DMSO-treated cells served as non-treated controls for cisplatin and Nutlin-3, respectively. Student's t-test was performed to compare the difference in the apoptotic cell score. *P<0.05 and **P<0.005 compared to the control. (B) Representative high content images of C666-1 cells stained with Annexin V-FITC for apoptosis (green), PI for viability (red) and Hoechst 33342 as counterstaining (blue) are shown. Imaging was conducted at 72 h post-treatment at magnification of x20. Annexin V(+)/PI(-) and Annexin V(+)/PI(+) cells were defined as apoptotic cells. (C) The effects of Nutlin-3 on the expression of apoptosis-related proteins in cisplatin-treated C666-1 cells. C666-1 cells were treated with cisplatin and/or Nutlin-3 (+) or 0.1% DMSO (-) for 24 h prior to analysis. The levels of p53 target apoptosis-related proteins are shown. β-actin was probed to monitor protein loading. Treatment of C666-1 cells with Nutlin-3 resulted in induction of p53 target apoptosis-related proteins p21, Mdm2, BAX and PUMA as compared to the vehicle control. FITC, fluorescein isothiocyanate; PI, propidium iodide.



Nutlin-3-adapted sublines (𝒳)	A	B	C	D	E	F	G	H
Number of passages at 0 μM	29	0	0	0	0	0	0	0
Number of passages at 10 μM	0	7	14	21	15	15	15	15
Number of passages at 20 μM	0	0	0	0	7	14	21	7
Number of passages at 40 μM	0	0	0	0	0	0	0	7
Total passages	29	7	14	21	22	29	36	29
IC_{50} Nutlin-3 of 𝒳	22.28 ±4.86	20.63 ±0.95	30.74 ±2.06	29.76 ±1.52	45.56 ±4.32	45.35 ±2.27	55.90 ±0.14	48.22 ±14.13
R value of 𝒳	1.00	0.93	1.38	1.34	2.04	2.04	2.51	2.16

Figure 5. Extended treatment with Nutlin-3 results in reduced sensitivity without emergence of p53 mutations. (A) The diagram shown is the experimental design to establish Nutlin-3-adapted NPC cell sublines. Nutlin-3-adapted C666-1 cell sublines were established by treatment of C666-1 cells with 10 to 40 μM Nutlin-3 over a period of up to six months. The sublines tested for p53 mutations are labeled (★). (B) Sensitivity of Nutlin-3-adapted C666-1 sublines to Nutlin-3 indicated by the concentration that inhibits cell viability by 50% (IC_{50}) following a 72-h treatment as quantified by MTS viability assay. The plot shown is representative mean IC_{50} values of three independent experiments carried out in triplicate. *P<0.05 and **P<0.005 compared to the parental control. (𝒳), Nutlin-3 adapted C666-1 sublines; R, resistance index indicates the degree of acquired resistance.

Nevertheless, suppression of endogenous p53 by lentiviral-based shRNA inhibited the effects of Nutlin-3 on C666-1 cells, thus indicating that the effects of Nutlin-3 on C666-1 cells were p53-dependent.

The anti-proliferative activity of cisplatin on NPC cells was found to be enhanced when combined with Nutlin-3. Our data indicate that Nutlin-3 sensitized the NPC cells to cisplatin-induced apoptosis. Similarly, these synergistic activities have been reported in neuroblastoma (46), gastric cancer (47) and ovarian cancer cells (48) and testicular germ cell tumors (49). Collectively, the enhancement of cisplatin-induced apoptotic cell death of NPC cells by Nutlin-3 was evidenced by the upregulation of p53, p21, Mdm2, BAX and PUMA expressions. These findings are in agreement with the notion that p53 promotes apoptosis by activating p53 pro-apoptotic targets.

Long-term treatment of cancer cells with increasing concentrations of Nutlin-3 could result in the emergence of Nutlin-3-resistant p53-mutated cells (36). In the present study, we found that although long-term exposure of wt p53 C666-1 cells to 10-40 μM Nutlin-3 in stepwise dose increments resulted in reduced sensitivity to Nutlin-3, the treatment did not result in the emergence of p53-mutated cells. This finding suggests that the acquisition of Nutlin-3 resistance in these cells could be due to other mechanisms. The reduction in sensitivity to Nutlin-3 in long-term treatment suggests that optimization in the clinical dose could be important to enhance the efficacy of Nutlin-3. However, such clinical implications have yet to be investigated fully.

In conclusion, we present evidence that Nutlin-3 is an effective p53 activator in NPC cells. Nutlin-3 was more toxic to NPC cells compared to non-malignant cells. The effects of Nutlin-3 on NPC cells were p53-dependent. In combination with cisplatin, Nutlin-3 promoted apoptosis induction in the NPC cells expressing wt p53. Collectively, the present study suggests that the potential use of inhibitors of p53-Mdm2 interaction should be explored further for the treatment of NPC.

Acknowledgements

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University of Crete

APPENDIX C

LIST OF PAPERS PRESENTED

Poster Presentation 1

Yee-Lin Voon, A Munirah, **PF Wong**, CO Leong, DP Lane and **ASB Khoo**. Combination of Nutlin-3 and cisplatin for the treatment of nasopharyngeal carcinoma. Poster presentation at 3rd NPC Research Day, Institute for Medical Research, Kuala Lumpur, 31st March 2014.

Abstract

COMBINATION OF NUTLIN-3 AND CISPLATIN FOR THE TREATMENT OF NASOPHARYNGEAL CARCINOMA

YL Voon[1], A Munirah[1], PF Wong[2], CO Leong[3], DP Lane[4], and ASB Khoo[1]

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Nasopharyngeal carcinoma (NPC), an epithelial squamous-cell head and neck carcinoma, is the third most common cancer among men in Malaysia and is highly prevalent among the Bidayuh natives of Sarawak. Tumour recurrence, distant metastasis, treatment toxicities and resistance remain as significant challenges in the clinical scenario.

p53 is a potential target for cancer therapy as its tumour suppressive activity can be stimulated to eradicate tumour cells. Mdm2 is an E3 ubiquitin ligase which targets p53 for degradation. Inhibition of p53-MDM2 interaction results in induction of p53, which, in turn could result in cell cycle arrest or apoptosis. The small molecule inhibitor of p53-MDM2 interaction, Nutlin-3, has been reported to be effective against cancers bearing wild-type p53. Previous work in our laboratory and reports by others suggest that p53 mutations are rare in NPC. Therefore, targeting p53-MDM2 interaction to induce p53 in NPC may offer a promising therapeutic strategy for NPC.

In this study, the effects of Nutlin-3 alone or in combination with Cisplatin, a common anti-cancer drug used to treat NPC, were evaluated on NPC and nasopharyngeal epithelial (NPE) cell lines using MTS assay and Western blotting. Combination therapy was analysed using CalcuSyn. Effects of the activation of the p53 pathway were assessed by expression analysis of p53 target genes, caspase enzyme activity and Annexin V apoptosis assay. We found that Nutlin-3 activates p53 and synergizes with Cisplatin in killing NPC cells. The combined treatment markedly reduced the IC50 of the individual compounds. Combination treatment also suppressed soft agar colony growth formation. Nutlin-3 sensitized NPC cells to Cisplatin-induced apoptosis, as determined by flow cytometry. In contrast, Nutlin-3 protected nasopharyngeal epithelial cells against Cisplatin-induced apoptosis. The effects of Nutlin-3 were rescued by knock down of p53, suggesting that the effects of Nutlin-3 on NPC cells were p53 dependent. Extended treatment of NPC cells with high doses Nutlin-3 did not result in the emergence p53 mutation in C666-1 cells. Taken together, our results suggest that combined treatment of Cisplatin with Nutlin-3 could potentially be useful for treatment of NPC.

University of Malaya

Combination of Nutlin-3 and Cisplatin for the Treatment of Nasopharyngeal Carcinoma

YL Voon[1], A Munirah [1], PF Wong[2], CO Leong[3], DP Lane[4] and ASB Khoo[1]**

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Abstract

Nasopharyngeal carcinoma (NPC), an epithelial squamous-cell head and neck tumour, is the third most common cancer among men in Malaysia and is highly prevalent among the Bidayuh natives of Sarawak. Tumour recurrence, distant metastasis, treatment toxicities and resistance remain as significant challenges in the clinical scenario. p53 is a potential target for cancer therapy as its tumour suppressive activity can be induced to eradicate tumour cells. Mdm2 is an E3 ubiquitin ligase which targets p53 for degradation. Inhibition of p53-MDM2 interaction results in induction of p53, which, in turn could result in cell cycle arrest or apoptosis. The small molecule inhibitor of p53-MDM2 interaction, Nutlin-3, has been reported to be effective against cancers bearing wild-type p53. Previous work in our laboratory and reports by others suggest that p53 mutations are rare in NPC. Therefore, targeting p53-MDM2 interaction to induce p53 in NPC may offer a promising therapeutic strategy for NPC. In this study, the effects of Nutlin-3 alone, or, in combination with Cisplatin, a common anti-cancer drug used to treat NPC, were evaluated for their effects on NPC and nasopharyngeal epithelial (NPE) cells using MTS assay and Western blotting. Combination therapy was analysed using CalcuSyn. Effects of the activation of the p53 pathway were assessed by expression analysis of p53 target genes and caspase enzyme activity. We found that Nutlin-3 activates p53 and synergizes with Cisplatin in killing NPC cells. The combined treatment markedly reduced the IC_{50} of the individual compounds. Combination treatment also suppressed soft agar colony growth formation. Nutlin-3 sensitized NPC cells to Cisplatin-induced apoptosis. In contrast, Nutlin-3 protected nasopharyngeal epithelial cells against Cisplatin-induced apoptosis. The effects of Nutlin-3 were rescued by knock down of p53, suggesting that the effects of Nutlin-3 on NPC cells were p53 dependent. Extended treatment of NPC cells with high doses Nutlin-3 did not result in the emergence p53 mutation in C666-1 cells. Taken together, our results suggest that combined treatment of Cisplatin with Nutlin-3 could potentially be useful for treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a major cancer in Malaysia. The cancer is strongly associated with the Epstein-Barr virus (EBV). Radiotherapy, chemotherapy, or a combination of both therapies is the standard treatment for NPC. Cisplatin is the most commonly used cytotoxic chemotherapeutic drug for NPC. However, local/loco-regional recurrence and metastases still remain as major clinical problems. Hence, discovery of new novel molecules is essential to further improve treatment outcome. Nutlins have been proposed as potential agents for p53-based therapies which kill cancer cells with wild type p53. Previous work in our laboratory and others have suggested that p53 is commonly wild type in NPC. In addition, adenoviral p53 gene therapy has been shown to kill NPC cells. However, the effects of nutlins on NPC have not been reported. We report our work on the effects of Nutlin-3 alone and in combination with Cisplatin in an EBV positive, p53-wild typed NPC cell line.

Materials & Methods

EBV positive C666-1 NPC cells as well as non tumorigenic (SV40T Ag immortalized nasopharyngeal NP69 and hTERT immortalized NP460 nasopharyngeal epithelial cells were used in this study. The p53 wild type status was confirmed by PCR and DNA sequencing (exon 2 – 11). All cell lines haboured wt p53 with codon 72 polymorphism. The MTS assay was used to assess the effects of Cisplatin and Nutlin-3 on cell viability. IC_{50} was calculated using Microsoft Office Excel. Calcusyn Biosoft was used for synergistic dose effect analysis. Soft agar colony formation assay was also performed to assess the effects on tumorigenecity of C666-1 cells. p53 knockdown was performed using a lentiviral-based shRNA and Western immunoblotting were carried out to investigate the mechanisms cell death.

Results

1. Nutlin-3 sensitizes nasopharyngeal carcinoma cells, but protects non-tumourigenic epithelial cells, to the cytotoxic effects of cisplatin

Figure 1 A

IC50 value of NPC C666-1, NP69 and NP460 cells to cisplatin and nutlin-3

Cells	Cisplatin μg/ml	Nutlin-3 μM
NP69	6.20 ± 0.93	33.69 ± 2.54
NP460	4.56 ± 1.11	22.96 ± 1.29
C666-1	9.69 ± 1.23	19.95 ± 0.93

Table 1A. IC50 value of monotherapy with Cisplatin or Nutlin-3.

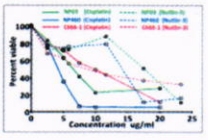


Figure 1A. Dose response of Cisplatin and Nutlin-3 on NPC(C666-1) and NPE (NP69 and NP460) cells.

Figure 1B

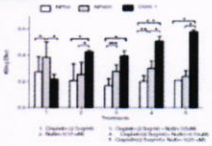


Figure 1B and Table 1B. The cytotoxic killing effects of Cisplatin, Nutlin-3 and the synergistic effects of the combination on C666-1, NP69 and NP460 cells. Drug interaction study was analyzed using the CalcuSyn Biosoft according to the method of Chou and Talalay. Interestingly, at several combination of doses, Nutlin-3 sensitized NPC cells while protecting nasopharyngeal epithelial cells to cisplatin-induced cytotoxicity.

Table 1B

Combination index (CI) values for drug combination effect of cisplatin and nutlin-3 in NP69, NP460 and NPC C666-1 cells

Cisplatin (ug/ml)	Nutlin-3 (μM)	NP69 Fa	NP69 CI	NP69 Description	NP460 Fa	NP460 CI	NP460 Description	C666-1 Fa	C666-1 CI	C666-1 Description
5.00	5.00	0.99 ± 0.00	2.52 ± 0.28	Antagonism	0.99 ± 0.00	2.97 ± 0.00	Antagonism	0.99 ± 0.00	1.98 ± 0.00	Antagonism
2.50	2.5	0.98 ± 0.00	3.86 ± 0.53	Antagonism	0.93 ± 0.01	3.31 ± 0.44	Antagonism	0.98 ± 0.00	2.17 ± 0.16	Antagonism
2.5	5	0.95 ± 0.00	5.62 ± 0.49	Antagonism	0.98 ± 0.00	4.05 ± 0.00	Antagonism	0.98 ± 0.00	1.98 ± 0.00	Antagonism
2.5	10	0.98 ± 0.00	4.82 ± 0.98	Antagonism	0.78 ± 0.06	6.84 ± 0.52	Antagonism	0.97 ± 0.00	2.52 ± 0.00	Antagonism
2.5	20	0.95 ± 0.00	5.27 ± 0.18	Antagonism	0.98 ± 0.00	4.05 ± 0.00	Antagonism	0.98 ± 0.00	2.52 ± 0.00	Antagonism

CI < 1.0 = Synergism CI = 1.0 = Antagonism CI > 1.0 = Additive effect

2. NPC cells treated with Cisplatin and Nutlin did not form colonies in soft agar

Figure 2A

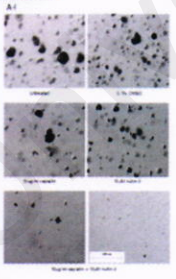


Figure 2B

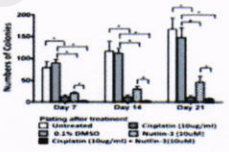


Figure 2A and 2B. Soft agar colony assay. C666-1 NPC cells which were treated with Cisplatin and Nutlin-3 were plated in soft agar. Post-treatment colony formation was markedly suppressed indicating that the drugs had successfully killed most cells.

3. Effects of nutlin-3 on NPC cells is p53 dependent

Figure 3A

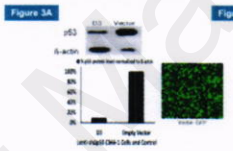
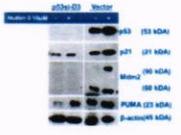


Figure 3B



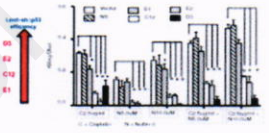


Figure 3A. Knockdown of p53 gene in C666-1 cells (lenti-sh.p53 C666-1) was successfully performed using lentiviral shRNA-p53si-D3 as shown on Western blot. (Transduction of lentiviral GFP vector showed efficiency). Figure 3B Knockdown of p53 rescued the induction of p53 and p53 target genes p21 and MDM2 by Nutlin-3. Fig 3C. Knockdown of p53 rescued NPC cells from the sensitization of the C666-1 NPC cells to cisplatin cytotoxity by Nutlin-3.

4. Nutlin-3 induced pro-apoptotic p53 target genes, BAX and PUMA

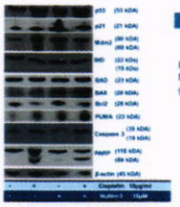


Figure 4

Figure 4. Disruption of p53-Mdm2 interaction by Nutlin-3 resulted in induction of p53 and p53 target genes, Mdm2, p21, BAX and PUMA.

5. Extended cultivation of C666-1 NPC cells in Nutlin-3 was not associated with the emergence of p53 mutations.

We attempted to generate Nutlin-3-resistant cells by extended propagation of C666-1 NPC cells in stepwise increasing concentrations (10, 20 and 40 μM) of Nutlin-3 over a period of six months. Extended pressure with Nutlin-3 resulted in a doubling of the IC_{50} to nutlin-3 but no mutations of the p53 gene (exons 2 – 11) were detected in these cells ($N^{+7/10}N^{+7/20}N^{+7/40}$ cells).

Figure 5

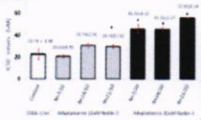
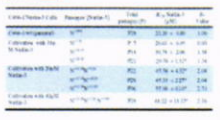


Table 5



Legend: $N^{+7/10}$ (7-passages with 10μM Nutlin-3), $N^{+14/10}$ (14-passages with 10μM Nutlin-3), $N^{+21/10}$ (21-passages with 10μM Nutlin-3), $N^{+15/10}N^{+7/20}$ (15-passages with 10μM, plus 7-passages with 20μM Nutlin-3), $N^{+21/10}N^{+14/20}$ (15-passages with 10μM, plus 14-passages with 20μM Nutlin-3), $N^{+15/10}N^{+7/20}N^{+7/40}$ (15-passages with 10μM, plus7-passages with 20μM, plus 7-passages with 40μM Nutlin-3).

Discussion and Conclusion

Nutlin-3, synergised with Cisplatin and increased the sensitivity of NPC cells, but not of non-tumourigenic nasopharyngeal epithelial cells, to Cisplatin with a markedly reduced IC50 of the compounds. Treatment of p53-wild type NPC cells with Nutlin-3 increased the expression of p53 and its target genes, p21 and MDM2, consistent with activation of the p53 pathway. These effects of Nutlin-3 on NPC cells were p53 dependent as knockdown of p53 rescued NPC cells to the effects of Nutlin-3. Nutlin-3 induced pro-apoptotic BAX and PUMA in NPC cells. Extended culture of NPC cells in Nutlin-3 resulted in modest increase in resistance but was not associated with emergence of p53 mutations.

References

1. Sun Y *et al.*, **An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma.** *Proc Natl Acad Sci U S A*,1992. 89(14): p. 6516-20.
2. Vassilev LT *et al.*, ***In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2.** *Science*, 2004. 303(5659): p. 844-8.
3. Weinrib *et al.*, **Cisplatin chemotherapy plus adenoviral p53 gene therapy in EBV-positive and –negative nasopharyngeal carcinoma.** *Cancer Gene Ther*, 2001. 8(5): p 352-60.

Acknowledgements

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Poster Presentation 2

Yee-Lin Voon, A Munirah, PF Wong, Roslina Husaini, Wayne Tiong Weng Ng, Tai Lin Chu, Chee Onn Leong, David P. Lane and Alan Soo Beng Khoo. Nutlin-3 sensitizes nasopharyngeal cancer cells to cytotoxic effects of cisplatin. Poster presentation at 17th NIH Scientific Seminar in Conjunction with NIH Research Week 2014, Institute for Health Management Bangsar, Kuala Lumpur, Malaysia, 24th – 25th November 2014.

University of Malaya

Abstract

NUTLIN-3 SENSITIZES NASOPHARYNGEAL CANCER CELLS TO CYTOTOXIC EFFECTS OF CISPLATIN

Yee Lin Voon[1,2], Munirah Ahmad[1], Pooi Fong Wong[2], Roslina Husaini[1] , Wayne Tiong Weng Ng[1], Tai Lin Chu[1], Chee Onn Leong[3], David P. Lane[4], and Alan Soo Beng Khoo[1]

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Small molecule inhibitors of p53-MDM2 interaction, such as nutlin-3, are known to be effective against cancers with wild type p53. Previous work in our laboratory and reports by others, suggest that p53 mutations are rare in nasopharyngeal carcinoma (NPC). Therefore, targeting p53-MDM2 interaction to induce p53 in NPC may be a potentially effective therapeutic strategy in NPC. In this study, the cytotoxic effects of nutlin-3 alone or in combination with cisplatin, a common anti-cancer drug used to treat NPC, was tested in an EBV positive NPC cell line with wild-type p53. Effects of the activation of the p53 pathway was assessed by expression analysis of p53 target genes, caspase enzyme activity and annexin V apoptosis assay. We found that nutlin-3 activates p53 and synergistically acts with cisplatin in killing the NPC cells. The combined treatment causes markedly reduced IC50 of the compounds and further suppressed soft agar colony growth formation. Nutlin-3 sensitized NPC cells to cis-platin induced apoptosis. The effects of nutlin-3 was rescued by knock down of p53, thus suggesting that the effect of nutlin-3 on NPC cells was p53 dependent. Our work suggests that combined treatment of cisplatin with nutlin-3 could potentially be useful for treatment of NPC.

Nutlin-3 Sensitizes Nasopharyngeal Cancer Cells To Cytotoxic Effects of Cisplatin

Yee Lin Voon[1,2], Munirah Ahmad[1], Pooi Fong Wong[2], Roslina Husaini[1], Wayne Tiong Weng Ng[1], Tai Lin Chu[1], Chee Onn Leong[3], David P. Lane[4], and Alan Soo Beng Khoo[1**]

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[2] Department of Pharmacology, Faculty of Medicine, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.
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Abstract

Small molecule inhibitors of p53-Mdm2 interaction, such as nutlin-3, are known to be effective against cancers with wild type p53. Previous work in our laboratory and reports by others, suggest that p53 mutations are rare in nasopharyngeal carcinoma (NPC). Therefore, targeting p53-Mdm2 interaction to induce p53 may be a potentially effective therapeutic strategy in NPC. In this study, the cytotoxic effects of nutlin-3 alone or in combination with cisplatin, a common anti-cancer drug used to treat NPC, was tested in an EBV positive NPC cell line with wild-type p53. Effects of the activation of the p53 pathway were assessed by expression analysis of p53 target genes, caspase enzyme activity and annexin V apoptosis assay. We found that nutlin-3 activates p53 and synergistically acts with cisplatin in killing the NPC cells. The combined treatment causes markedly reduced IC_{50} of the compounds and further suppressed soft agar colony growth formation. Nutlin-3 sensitized NPC cells to cisplatin induced apoptosis. The effects of nutlin-3 was rescued by knock down of p53, thus suggesting that the effect of nutlin-3 on NPC cells was p53 dependent. Our work suggests that combined treatment of cisplatin with nutlin-3 could potentially be useful for treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a major cancer in Malaysia. The cancer is strongly associated with the Epstein-Barr virus (EBV). Radiotherapy, chemotherapy, or a combination of both therapies is the standard treatment for NPC. cisplatin is the most commonly used cytotoxic chemotherapeutic drug for NPC. However, local/loco-regional recurrence and metastases still remain as major clinical problems. Hence, discovery of new novel molecules is essential to further improve treatment outcome. Nutlins have been proposed as potential agents for p53-based therapies which kill cancer cells with wild type p53. Previous work in our laboratory and others have suggested that p53 is commonly wild type in NPC. In addition, adenoviral p53 gene therapy has been shown to kill NPC cells. However, the effects of nutlins on NPC have not been reported. In our work, we studied the effects of nutlin-3 alone and in combination with cisplatin in an EBV positive, p53-wild typed NPC cell line.

Materials & Methods

An EBV positive NPC cell line, C666-1 was used in this study. Its p53 wild type status was confirmed by PCR and DNA sequencing (exon 2–11). The MTS assay was used to assess the effect of cisplatin and Nutlin-3 on cell viability. IC_{50} was calculated using Microsoft Office Excel. In the soft agar colony formation assay, cells were treated for 48 h, then seeded in 6-well plate in triplicates. Colony formation was observed and recorded at day 7, 14 and 21. The apoptotic effect of Nutlin-3 or/and cisplatin on C666-1 cells was determined by Annexin V High Content Analysis and Caspases 3/7 assay. p53 knockdown was performed using a lentiviral-based shRNA. Western blot analysis was carried out to determine the target protein expressions level and the p53 knockdown efficiency. β-actin served as a loading control. Calcusyn Biosoft was used for synergistic dose effect analysis.

Results

1 Nutlin-3 activates p53 in C666-1 NPC cells

Fig.1

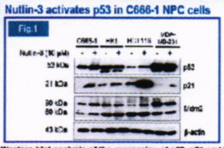


Fig.1. Western blot analysis of the expression of p53, p21 and Mdm2 proteins in cells treated with and without 10 μM Nutlin-3 for 24 hours. β actin served as a loading control. Wild type p53 HCT116, mutant p53 HK1 and MDA-MD231 cell lines were used as controls. Treatment of C666-1 cells with 10μM Nutlin-3 increased the protein levels particularly the p21 level, thus, suggesting that the treatment with nutlin-3 activates p53 pathway in this cell line. In cell lines with mutant p53, treatment of nutlin 3 showed no effects on the p53 and Mdm2 levels with only very minimal increase in p21.

2 Combined treatment of Nutlin-3 and cisplatin markedly reduced the IC_{50} values

Fig.2A, Fig.2B

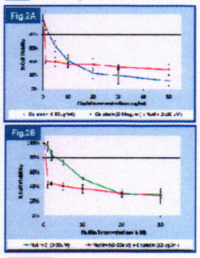


Fig.2. C666-1 NPC cell viability by MTS assay (A) after treatment with cisplatin alone (0-50 μg/ml) and in combination with Nutlin-3 (10μM) and (B) after treatment with Nutlin-3 alone (0-30 μM) and in combination with cisplatin (10μg/ml) at 48 h time point. A combined treatment using both drugs on C666-1 cells had markedly reduced the IC_{50} (Table.1)

Table 1

IC_{50} values for Cis-platin, nutlin-3 and combination of both for NPC C666-1 cells	
	IC_{50}
Cis-platin alone (0-50 μg/ml)	7.97 ± 1.84
Cis-platin(0-30μg/ml) + Nutlin-3 (10 μM)	1.1 ± 0.26
Nutlin-3 alone (0-30 μM)	10.2 ± 0.87
Nutlin-3 (0-30 μM) + Cisplatin (10 μg/ml)	0.83 ± 0.13

Table.1. IC_{50} values for cisplatin, Nutlin-3 and in combination of both for NPC C666-1 cells.

3 Treatment with nutlin-3 suppresses colony formation

Fig.3A

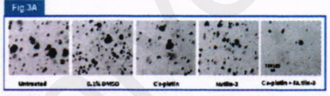
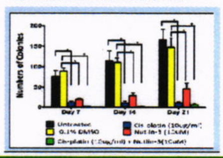


Fig.3B

Fig.3. Effects of Nutlin-3 and/or cisplatin on soft agar colony formation. (A) C666-1 cells were treated for 48 h. After treatment, cells were then sub-cultured into 0.3% agarose. 0.1% DMSO served as vehicle control for Nutlin-3. (B) A single colony with a diameter >60μm imaged at high magnification. (C) Colonies were scored using an Image Pro Plus AMS version 6.3 software. Statistical analysis, paired T test was performed to compare the difference in the mean of numbers of colonies scored, * indicate p< 0.05.

Fig.3C



4 Nutlin-3 sensitized NPC cells to cisplatin induced apoptosis

Fig.4A, Fig.4B, Fig.4C

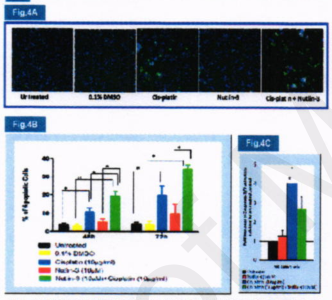


Fig.4 Evaluation of apoptotic effects induced by treatment with cisplatin and nutlin-3. (A) After treatment for 48h and 72h, C666-1 cells were stained with AnnexinV-FITC for apoptosis detection **(Green)**, Propidium Iodide for viability (Red) and Hoechst 33342 as counter stain (Blue). **(B)** The percentage of apoptotic cells were calculated using Metamorph. Treatment with cisplatin (10mg/ml) alone and in combination with nutlin-3 (10uM) was found to significantly increase apoptosis while nutlin-3 alone did not. T-test was performed to compare between treatments in which * indicated significance (p<0.05). **(C)** Caspases 3/7 enzyme activities were measured after 48h of treatment. cisplatin but not Nutlin-3 induced caspases 3/7 activation.

5 Establishment of p53 knockdown C666-1 NPC cells

Fig.5A

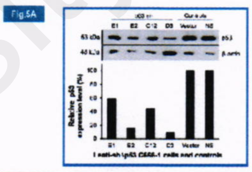


Fig.5B

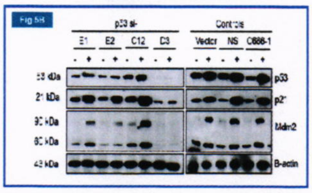


Fig.5. Western blotting analysis and measurement of p53 knockdown efficiency in C666-1. 4 constructs of Lentiviral p53 shRNA (p53si E1, E2, C12 and D3) were tested. pLKO.1-puro empty vector, non-specific (NS) shRNA and/or untransduced cells were used as controls. At 72h post transduction, cells were harvested and subjected to western blot assay. **(A)** Among the 4 constructs, p53si-D3 and E2 significantly reduced the p53 protein level. Quantitation using densitometer software showed that p53si D3 exhibited more than 80% knockdown of endogenous wild type p53 protein in C666-1 cells. (B) Western blot analysis of the expressions of p53 target genes in the presence or absence of 20 μM Nutlin-3 for 24 h in the p53 knockdown cells. β-actin served as a loading control. The results showed that pLKO or NS did not affect the expression of endogenous p53 level.

6 p53 gene silencing with lenti-shΔp53 reduces sensitivity of NPC to Nutlin-3.

Fig.6A, Fig.6B

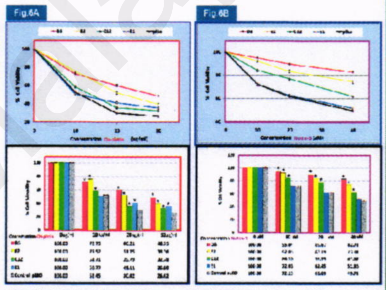


Fig.6. Effects of knockdown of p53 on sensitivity to nutlins. Lenti-shΔp53 C666-1 and control cells were treated with increasing concentrations of cisplatin 0-30 ug/ml (A) and nutlin-3 (0-40 μM) (B) at 72 h time point. Cell viability was measured by MTS. Error bars represent mean ± S.D. for three independent experiments with 9 data points. As shown in these diagrams, p53 gene silencing with lenti-shΔp53 reduces sensitivity of NPC to both Nutlin-3 and cisplatin.

7 Nutlin-3 synergises with cisplatin in effectively killing NPC cells in a p53 dependent manner

Table 2

Cisplatin (ug/ml)	Nutlin-3 (uM)	Fraction affected (fa)	Combination Index (CI)	Description
1.25	1.25	[illegible]	[illegible]	Synergism
[illegible]	[illegible]	[illegible]	[illegible]	Synergism
[illegible]	[illegible]	[illegible]	[illegible]	Synergism
[illegible]	[illegible]	[illegible]	[illegible]	Synergism
[illegible]	[illegible]	[illegible]	[illegible]	Synergism

Fig.7A, Fig.7B

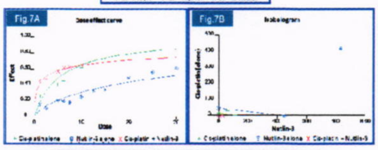


Fig.7C

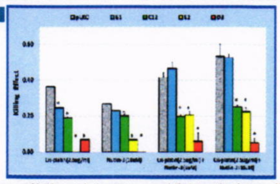


Fig.7. Drug combination study to assess synergistic interaction between Nutlin-3 and cisplatin in NPC. C666-1 NPC cells were treated with combinations of cisplatin and Nutlin-3 at a fixed concentration ratio. Cell viability/killing effect was then determined by MTS. Multiple drug dose–effect calculations and the combination index (CI) plots were generated using the CalcuSyn Biosoft software. **(A)** Dose effect curve. **(Table 2)** Fraction affected (fa) and CI values. CI<1 indicated synergism. (B) Isobologram demonstrated that the combination of both drugs showed synergistic effects at ED50 and ED75. (C) The synergistic effects of combined treatment were also tested in lenti-shΔp53 C666-1 cells versus control cells. Knockdown of p53 significantly reduced the synergistic effects of nutlins and cisplatin suggesting that the effect is dependent on p53.

Discussion and Conclusion

Treatment of p53-wild type NPC cells with Nutlin-3 increased the expression of p53 and its target genes, p21 and Mdm2, consistent with activation of the p53 pathway. Nutlin-3, synergised with cisplatin and increased the sensitivity of NPC cells to cisplatin with a markedly reduced IC_{50} of the compounds. The results were consistent with the effects of Nutlin-3 and cisplatin in colony formation assays. Furthermore, high content analysis showed that Nutlin-3 sensitized NPC cells to cisplatin induced apoptosis. Finally, knockdown of p53 rescued the effects of Nutlin-3 on NPC cells. Our work shows that Nutlin-3 activates p53 in p53-wild type NPC cells, and sensitized the cells to cisplatin induced apoptosis in a p53-dependent manner. Combined treatment of cisplatin with Nutlin-3 may be potentially useful for therapy of NPC.

References

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2. Vassilev LT *et al.*, *In vivo* **activation of the p53 pathway by small-molecule antagonists of Mdm2.** *Science*, 2004. 303(5659): p. 844-8.
3. Weinrib *et al.*, **cisplatin chemotherapy plus adenoviral p53 gene therapy in EBV-positive and –negative nasopharyngeal carcinoma.** *Cancer Gene Ther*, 2001. 8(5): p 352-60.

Acknowledgements

This work was supported by a grant from the Ministry of Health Malaysia (JPP-IMR 06-059 (NPC4). We thank KW Lo (Chinese University of Hong Kong) for kindly providing the NPC C666-1 cells and Addgene for Lentiviral packaging and envelope plasmids.

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Oral Presentation 1

Yee-Lin Voon, Munirah Ahmad, **Pooi-Fong Wong**, Chee-Onn Leong, David Phillip Lane, **Alan Soo-Beng Khoo**. p53-Mdm2 Interaction Targeted Therapy by Nutlin-3 on Nasopharyngeal Carcinoma Cells. Oral presentation by Voon Yee Lin at Journal Club Department of Pharmacology, Faculty of Medicine, University of Malaya, Malaysia, 1st March 2013.

Oral Presentation 2

Yee-Lin Voon, Munirah Ahmad, **Pooi-Fong Wong**, Chee-Onn Leong, David Phillip Lane, **Alan Soo-Beng Khoo**. Translation Research in Nasopharyngeal Carcinoma. Oral presentation by Dr Alan Khoo at 2nd Nasopharyngeal Carcinoma Research Day, University of Malaya, Malaysia, 4th March 2013.

Oral Presentation 3

Yee-Lin Voon, Munirah Ahmad, **Pooi-Fong Wong**, Chee-Onn Leong, David Phillip Lane, **Alan Soo-Beng Khoo**. Development of the Therapeutic Agents for Treatment of Nasopharyngeal Carcinoma. Oral presentation by Dr Alan Khoo at 1st National Conference for Cancer Research in Conjunction with 5th Regional Conference on Molecular Medicine, Kuala Lumpur, 8th – 10th November 2013.

Oral Presentation 4

Yee-Lin Voon, Munirah Ahmad, **Pooi-Fong Wong**, Chee-Onn Leong, David Phillip Lane, **Alan Soo-Beng Khoo**. p53-Mdm2 Interaction Targeted Therapy by Nutlin-3 on Nasopharyngeal Carcinoma Cells. Oral presentation by Voon Yee Lin at Candidature Defence, Department of Pharmacology, Faculty of Medicine University of Malaya, Malaysia, 26th March 2014.

Oral Presentation 5

Yee-Lin Voon, Pooi-Fong Wong, Alan Soo-Beng Khoo. p53-Mdm2 Interaction Targeted Therapy by Nutlin-3 on Nasopharyngeal Carcinoma Cells. Oral presentation by Voon Yee Lin at International Postgraduate Research Awards Seminar (InPRAS2016), University of Malaya, Malaysia, 7th – 8th March 2016.

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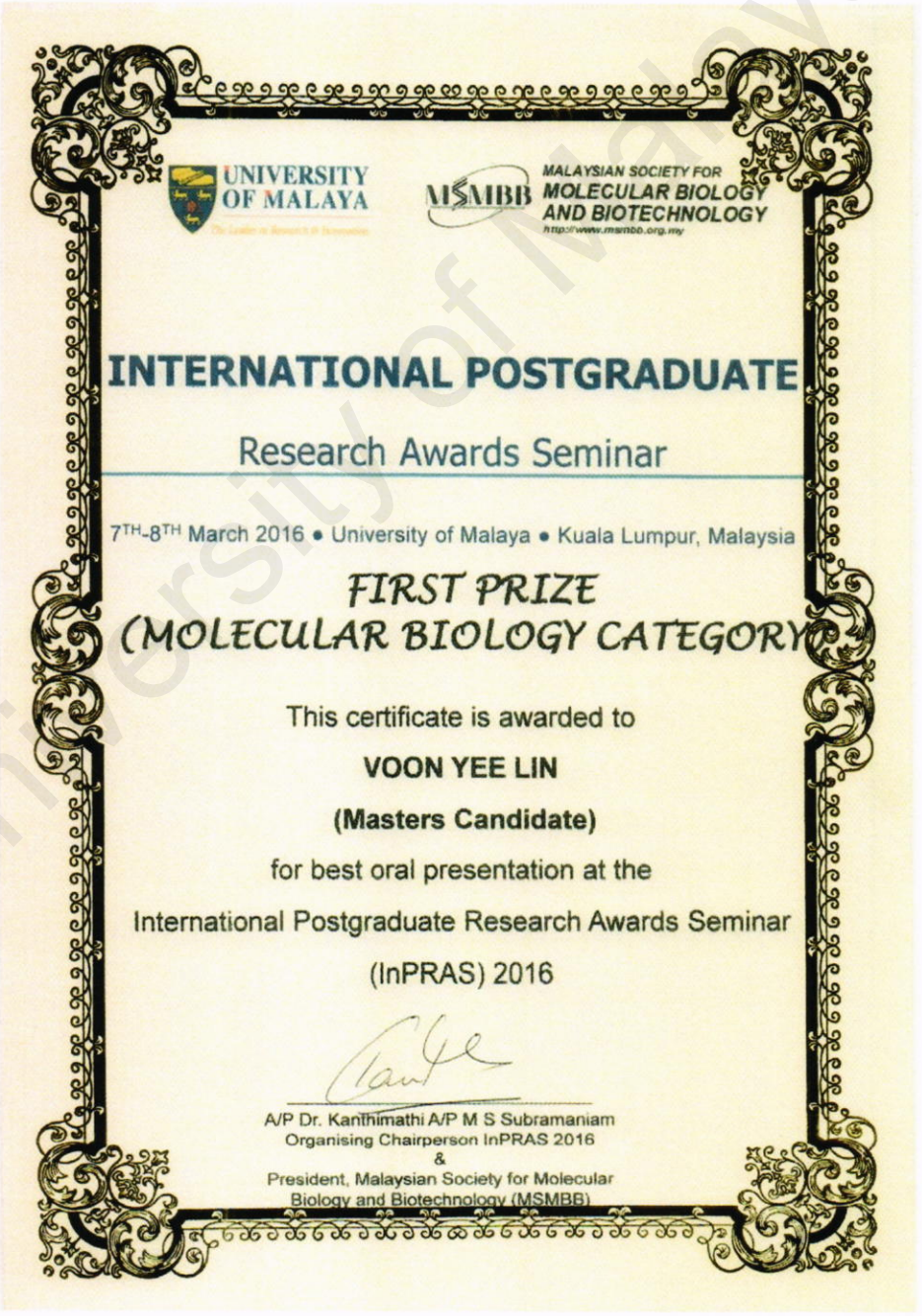
APPENDIX D

LIST OF AWARDS

Best Oral Presentation
1st Prize for Molecular Biology Category, Masters Candidate

Awarded for:
p53-Mdm2 Interaction Targeted Therapy by Nutlin-3 on Nasopharyngeal Carcinoma Cells

Awarded by:
International Postgraduate Research Awards (InPRAS2016)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INTERNATIONAL POSTGRADUATE

Research Awards Seminar

7TH-8TH March 2016 • University of Malaya • Kuala Lumpur, Malaysia

FIRST PRIZE
(MOLECULAR BIOLOGY CATEGORY)

This certificate is awarded to

VOON YEE LIN

(Masters Candidate)

for best oral presentation at the

International Postgraduate Research Awards Seminar

(InPRAS) 2016

A/P Dr. Kanthimathi A/P M S Subramaniam
Organising Chairperson InPRAS 2016
&
President, Malaysian Society for Molecular Biology and Biotechnology (MSMBB)

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