Investigating the Molecular Mechanism of Novel Quinuclidinone Derivatives in Lung Cancer Cells with Different p53 Status

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Eroica Soans
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This dissertation titled
Investigating the Molecular Mechanism of Novel Quinuclidinone Derivatives in
Lung Cancer Cells with Different p53 Status

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ABSTRACT

EROICA SOANS, Ph.D., August 2010, Chemistry and Biochemistry

Investigating the Molecular Mechanism of Novel Quinuclidinone Derivatives in Lung Cancer Cells with Different p53 Status (134 pp.)

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Most chemotherapeutics affect normal cells as much as cancer cells leading to many undesirable side effects. One major goal is to develop cancer targeted chemotherapeutics that minimize side effects. Previously, novel quinuclidinone derivatives that cause cytotoxicity in human non-small lung carcinoma epithelial cells null for p53 (H1299) were reported. Here the mechanism involved in this cytotoxic effect was investigated. Quinuclidinone derivatives 8a and 8b induced cytotoxicity mainly through apoptosis of lung cancer cells independent of p53 status with induction of S-phase arrest. Importantly, they cause lower cytotoxicity and minimal apoptosis in normal lung epithelial cells (NL-20). This effect could be related to the elevated sphingomyelinase activity we detected in cancer cells compared to normal cells.

Furthermore, we observed that different sphingomyelinase isoforms are involved in 8a and 8b induced cytotoxicity of cancer cells. Sphingomyelinase initiated apoptosis through ceramide up-regulation with increased phosphorylation of JNK. The extrinsic and intrinsic apoptosis pathways are involved in 8a and 8b induced apoptosis as seen with the processing of procaspase 8, up-regulation of bax, cytosol cytochrome c and caspase 9, and down-regulation of bcl-2 proteins. Also, the derivatives induced p53 dependent apoptosis through the mitochondrial pathway. Gene array analysis implicated the
involvement of the TNF receptor super family. Together these results show that the mechanism by which quinuclidinone derivatives provoke cytotoxicity in lung cancer cells is through sphingomyelinase dependent apoptosis involving both extrinsic and the intrinsic pathways.

Approved: 

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CHAPTER 1: INTRODUCTION

1.1 Non Small Cell Lung Carcinoma

Cancer is one of the principal causes of death in the United States and in other countries (Jemal et al., 2005). It is a complex disease studied since 1500 b.c. and is simply defined as uncontrolled growth of normal cells. This uncontrolled growth is mainly due to acquired genetic mutations leading to defects in regulatory systems that oversee normal cell proliferation and homeostasis. Cancer is characterized by the type of cells affected and tissue it invades (Evan & Littlewood, 1998; Hanahan & Weinberg, 2000). The principle types are carcinomas (epithelial cells), sarcomas (connective tissue cells) and lymphomas or leukemia (hematopoietic or blood cells) (Berman, 2004; Golub et al., 1999; Oien & Evans, 2008). Among all cancers, carcinomas are the most common form and lung cancer has been identified as the most lethal form of carcinoma as it causes the highest number of deaths in both males and females (Table 1) (Jemal et al., 2008).

Lung cancer has been on a rise since the 1930’s. It is classified into two main types, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC, large cell carcinoma), based on the invading cell morphology. The more prevalent type is NSCLC reported for more than 80 % of lung cancer cases. NSCLC is further classified, depending on the histology, into 3 major types: adenocarcinoma (most common), squamous cell carcinoma and large cell carcinoma. Smoking and secondary smoke inhalation are root causes of this disease. Other contributing factors are chronic pulmonary disease, dietary intakes lacking fruits and vegetables, arsenic, asbestos radon, chromium, nickel, air pollution, and family history.
Table 1

*Cancer mortality statistics in both genders as observed in United States and adapted from Jemal et al 2008.*

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>% Estimated Deaths Male</th>
<th>% Estimated Deaths Females</th>
</tr>
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<tr>
<td><strong>Lung</strong></td>
<td>30</td>
<td>26</td>
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<tr>
<td>Prostate</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>Breast</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Colon</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Pancreas</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Leukemia</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Others</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td><strong>All Sites</strong></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The survival mean of NSCLC patients is less than 5 yrs for stage 1 (early detection) patients however not more than 15 % can survive till 5 years (Alberg & Samet, 2003; Herbst, Heymach, & Lippman, 2008; Walker, 2008). Approximately 70 % of NSCLC have a mutation in the tumor suppressor protein, p53, which is associated with high risk and poor prognosis. Alterations in the *TP53* gene that have been attributed to NSCLC tumorigenesis include the prevalent G to T transversion in the coding region and also
some C to T transitions in the CpG dinucleotide regions in smokers, while in non-smokers it is mainly G to A transition with some of G to C transversions. In many cases a deletion of chromosome region 17p13.3 causes loss of wild type p53 allele which is also a contributing factor for tumor progression (Ahrendt et al., 2003; Herbst et al., 2008; Hwang et al., 2003; Marchetti et al., 1993).

1.2 p53 is the ‘guardian of the genome’

p53 was first described as an oncogene in 1979 (DeLeo et al., 1979) but ten years later reclassified as a tumor suppressor protein (Baker et al., 1989; Finlay, Hinds, & Levine, 1989) that is mutated in most cancers, especially in NSCLC. It is involved in many cellular processes that help in controlling DNA damage and thereby helps in maintaining the cellular homeostasis. Some of these cellular processes in which p53 is involved include apoptosis, cell cycle arrest, development, differentiation, gene amplification and cellular senescence (Oren & Rotter, 1999). It can be activated in the cell by varied stimuli such as UV radiation, gamma radiation, hypoxia, virus, chemotherapeutics, heat shock or over-expression of an oncogene. Upon activation, whether through protein up-regulation or post-translational modification (e.g. phosphorylation), p53 mainly functions as a transcription factor that up-regulates genes that are involved in cell cycle regulation and apoptosis. Some of the up-regulated genes identified in cell cycle regulation are p21 and Gadd45a (growth arrest and DNA damage inducible protein 45 alpha), and in apoptosis are bcl-2 family of genes like bax (bcl-2 associated X protein), and BH3 domain related genes, like puma (p53 up-regulated
modulator of apoptosis) and noxa (Latin word for damage). p53 also can cause transcription repression of certain genes like bcl-2 (B-cell CLL/lymphoma 2), bcl-X and survivin (Evan & Littlewood, 1998; Milne, Campbell, Campbell, & Meek, 1995; Vogelstein, Lane, & Levine, 2000; Vousden & Lu, 2002; Wei et al., 2006).

**Fig. 1: p53: structure, cellular stress and cellular activities.** p53 protein has a transactivation domain (TAD), DNA binding domain, tetramerization domain (TET) nuclear localization sequence (NLS) and nuclear export signal sequence (NES). p53 is activated by many stimuli (intracellular and extracellular) and transactivates downstream targets that carry out different cellular processes.
To carry out these cellular functions the p53 protein has different domains. Through the tetramerization domain, p53 protein forms active tetramers. The nuclear localization domain helps in transport of the active p53 into the nucleus from the cytoplasm. The transactivation domain is involved in recruiting and interacting with transcription factors and the DNA binding domain binds to consensus sequence of 10 bp on the DNA. p53’s interaction with its response elements present on the corresponding DNA regulates either increase or decrease in transcription of that particular gene. (Fig. 1) (Evan & Littlewood, 1998; Kern et al., 1991; Milne et al., 1995; Vogelstein et al., 2000; Vousden & Lu, 2002; Wei et al., 2006).

p53 is involved in many diverse functions that its functional absence is contributed to NSCLC poor prognosis and may be involved in resistance to chemotherapy, as many chemotherapeutics work well in the presence of it. NSCLC is most often detected in the advance stage where the only line of therapy available is chemotherapy. Therefore many efforts are being made to develop chemotherapeutics that work independent of p53 status (Lee & Bernstein, 1995; Mueller & Eppenberger, 1996; Vogelstein et al., 2000).

1.3 Chemotherapy

Chemotherapeutics, first discovered in the 1940s, are cytotoxic or cytostatic chemical agents used to treat a disease. The anti-cancer chemotherapeutics were classically differentiated as alkylating agents, e.g. mustard gas (the first documented
chemotherapeutic), cyclophosphamide, cisplatin and carboplatin; anti-metabolites, e.g. gemcitabine, fluorouracil; plant alkaloids, e.g. vinblastin, taxol; antitumor antibiotics, e.g. doxorubicin; topoisomerase inhibitors, e.g. etoposide; and mitosis inhibitors e.g. paclitaxel. The mode of action of most of these are to interfere with nucleic acid synthesis or metabolism by cross linking with the DNA, forming adducts, intercalating with DNA strands, inhibition of topoisomerases I, II and finally by interrupting purine and pyrimidine biosynthesis (Chabner & Roberts, 2005; Colombo, Gunnarsson, Iatropoulos, & Brughera, 2001; DeVita & Chu, 2008; Espinosa, Zamora, Feliu, & Gonzalez Baron, 2003; Hurley, 2002; Papac, 2001). The recent trend is to identify new specific targets other than nucleic acids to overcome the possible resistance that most cancers develop against chemotherapeutics and lower the toxicity towards normal tissues that lead to many side effects observed with the therapy. These could include targets against intra and extracellular receptors, endoplasmic proteins, mitochondrial proteins, cytoplasmic proteins or the extracellular matrix proteins (Chabner & Roberts, 2005; DeVita & Chu, 2008; Espinosa et al., 2003; La Porta, 2004).

Chemotherapy is either given as the first line therapy or as an adjuvant or as combinatorial drug. In most cancers especially in early stages of NSCLC, it is given as adjuvant therapy before surgery, and as a combinatorial drug with radiation in locally advanced NSCLC. Sometimes chemotherapy is the only therapy given in advanced cancers, especially NSCLC (DeVita & Chu, 2008; Furuse et al., 1999; Ginsberg & Rubinstein, 1995; Joensuu, 2008; Marino, Pampallona, Preatoni, Cantoni, & Invernizzi, 1994). However, NSCLC has the propensity to resist different chemotherapy regimens,
making prognosis poor, therefore efforts have been made to develop better and efficient chemotherapeutics (Furuse et al., 1999; Ginsberg & Rubinstein, 1995; Marino et al., 1994).

In the 1970s and 1980s the first line of chemotherapeutics against NSCLC was platinum derivatives. In the 1990s, new drugs were developed such as irinotecan, paclitaxel, docetaxel, vinorelbine, gemcitabine, topotecan, and amurubicin. These derivatives were also used in combination against stage IV NSCLC (metastatic advanced lung cancer). These agents show a modest improvement in prolonging survival and quality of life of patients as shown by past randomized trials. All these drugs that are used as current standard chemotherapeutic treatment belong to classical group of chemical drugs that affect the nucleus by either binding to the DNA (cisplatin or carboplatin) or by acting as topoisomerase I or II inhibitors (irinotecan, topotecan and amurubicin) or mitotic inhibitors (paclitaxel or docetaxel) or nucleoside analog (gemcitabine). Though sometimes they are the only therapy available, they cause many side effects which affect the quality of life of the patients. These side effects mainly include hematological symptoms with neutropenia. Most of these drugs help in disease control for a short time, however the side effects that increase with prolonged treatment are leading many scientists to develop therapeutics that increase the time of disease free-state and reduce the side effects.

The current trends are moving into the development of therapies that target cancer specific cellular molecules, such as proteins, lipids and mitochondrial DNA rather than the traditional nuclear DNA thereby not affecting the normal cells as much. These
therapies require a molecular profile of tumors for selection of therapy to be used. One such target oriented agent now under clinical trial that has been approved for patients that have tyrosine kinase mutation in certain cancers is EGFR (epidermal growth factor receptor) inhibitor (Belani, 2010; Chen et al., 2002; Fukuoka, 2000; Kato & Saijo, 2002; Owonikoko, Ramalingam, & Belani, 2010; Ramanathan & Belani, 1997). In many cancers, including NSCLC, p53 is mutated and therefore makes it an interesting drug target (Almazov, Kochetkov, & Chumakov, 2007).

1.4 PRIMA-1 restores mutant p53 function

Most chemotherapeutics work better in the presence of wild type p53 but efforts are being made to develop small molecules that restore mutant p53 function in cancer cells (Lee & Bernstein, 1995; Mueller & Eppenberger, 1996; Weller, 1998). Some examples of these small molecules that show this activity are CP-31398, a pyrimidine derivative, which restores mutant p53 function and stabilizes wild type p53 in glioma cells (W. Wang, Takimoto, Rastinejad, & El-Deiry, 2003; Wischhusen, Naumann, Ohgaki, Rastinejad, & Weller, 2003), ellipticineum, small chemical molecules, used in colon, CNS and kidney cell lines having a p53 mutation (Shi et al., 1998) and WR105 works against temperature sensitive Met 278 mutation of p53 by restoring its wild type conformation in esophageal cancers (North, Pluquet, Maurici, El-Ghissassi, & Hainaut, 2002). The most interesting of them all is the PRIMA-1 derivative that has been shown to restore p53 function in many tumor types (Bykov, Issaeva, Selivanova, & Wiman, 2002; Bykov, Issaeva, Shilov et al., 2002).
PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a low molecular weight compound, 2, 2-bis (hydroxymethyl)-1-azabicyclo [2,2,2] octan-3-one (Fig. 2). It restores mutant p53 activity in cell lines expressing His 175 mutant p53. This mutation causes major structural defect, as wild type Arg 175 is required for stabilizing the DNA binding domain structure and is frequently mutated and linked to improper folding. PRIMA-1 acts by forming adducts with the thiols present on p53 through covalent modification, allowing for the correct folding of the p53 protein so that it can bind DNA and transactivate downstream genes (Bykov, Issaeva, Selivanova et al., 2002; Bykov, Issaeva, Shilov et al., 2002; Cho, Gorina, Jeffrey, & Pavletich, 1994; Lambert et al., 2009).

In breast cancer cells carrying this p53 mutation, PRIMA-1 is shown to increase the promoter activity and protein expression of bax and PUMA, known transcriptional targets of p53, and lower the expression of cJun and phosphorylated cJun. This suggests that PRIMA-1 causes apoptosis through bax and PUMA but not JNK signaling (T. Wang, Lee, Rehman, & Daoud, 2007). In NSCLC cells expressing the His 175 mutation and colon cancers with mutant p53, PRIMA-1 caused G2 arrest with up-regulation in p21 and Gadd45a proteins (known transcriptional targets of p53) along with the induction of JNK signaling pathway, but did not have any effect on Fas, bcl-2 related proteins, caspase 8, caspase 9 or caspase 3 (Y. Li, Mao, Brandt-Rauf, Williams, & Fine, 2005). These studies taken together suggest that PRIMA-1 molecular mechanisms are cell type dependent. Most importantly, this intriguing molecule works at very high concentration which would not have desirable pharmacokinetics and toxicity profile. Therefore, it is important to
develop similar or better compounds. When PRIMA-1 was first recognized as potential anti-cancer drug, there was no structural activity analysis. This led to the development of quinuclidinone derivatives from PRIMA-1 in collaboration with Dr Stephen Bergmeier’s research group in the department of Chemistry at Ohio University.

1.4 Structural activity analysis of novel quinuclidinone derivatives.

The rationale for designing many derivatives from the lead compound PRIMA-1 (now onwards to be referred as derivative 4) was to understand the functional importance of each chemical group attached to the lead compound to help in developing better and more efficient compounds with either substitution or modification of the original structure. In derivative 6 the ketone was substituted to a diol, diol was converted to diacetates in derivative 7 and then a series of acetals were prepared by condensing the diol with appropriate carbonyls (Fig. 2).

In 2006, Malki et al showed, using the MTT cell viability analysis that detects activity of the mitochondrial enzyme present in viable cells, that 4 mM concentration of the lead compound 4, as expected, showed lower viability of the tumor cell line tested (H1299, NSCLC cell line having null p53) than the other derivatives. Derivative 6 had no effect indicating that the ketone group is functionally important and derivative 7 had similar activity as derivative 4 indicating the hydroxyl groups were not as functionally significant as the ketone. Most interesting, the two acetals, derivatives 8a and 8b, had improved activity as compared to compound 4. Also, when H1299 cells were treated with the 8a and 8b derivatives then with gamma radiation, they showed additive and
synergistic effect as compared to the lead compound. Drug response curve analysis showed both 8a and 8b, whether used alone or in conjunction with gamma radiation, followed dose dependence curve, wherein both 8a and 8b effect increased with increase in dose. The GI\textsubscript{50} for 8a and 8b was reported as 17.26 \(\mu\text{M}\) and 19.21 \(\mu\text{M}\), respectively. The molecular weight of 8a and 8b are 273.33 and 225.28, respectively.

Fig. 2: Chemical structures of quinuclidinone derivatives. PRIMA-1 (derivative 4) restores p53 activity at very high concentration. Derivatives 8a (benzylidine) and 8b (acetonide) were selected for further studies as it shows higher activity than PRIMA-1 in H1299 cells.

$\text{indicates the compound number as published by Bykov et al in 2002; } \%\text{ indicates compound number given by Bergmeier’s group; } #\text{ indicates compound number as published by Malki et al in 2006.}$
More derivatives were prepared from 8a and 8b to understand the functional significance of each group on the structure. Derivative 8c (acetal of cyclohexane carboxaldehyde) was prepared to determine if the aromatic ring of 8a was essential. 8c has a lesser effect as compared to 8a but a similar effect as compound 4. Derivative 8d (acetal derived from cyclohexanone) shows lowered activity as compared to both 8b and 4. Derivative 8e was prepared to determine if the aromatic ring on 8a could be shifted and if it would retain potency but this compound has lowered effect as compared to 8a and similar potency as compared to 4. Derivatives 8f and 8g have substituted aromatic rings and both show much lower effect as compared to derivative 8a and lead compound 4 (Malki et al., 2006).

Derivatives 8a and 8b were selected as the lead quinuclidinone derivatives for further studies since they were the most effective compared to the other derivatives studied. Since PRIMA-1 works well at very high concentration a lower dose of 100 µM was selected to do a comparison study between all derivatives prepared. For this preliminary study to understand the effectiveness of the derivatives in addition to H1299 used in the previous study, H1299 p53 +/- (over-expressing p53) and NL-20 (normal lung epithelial) cells were also included. H1299 p53 +/- were included to study if the drugs were p53 dependent or independent in their effectiveness. While NL-20 cells were incorporated to understand the modality of the derivatives, specifically if they were as effective on normal cells as they were on cancer cells. From Fig. 3, it is obvious that derivatives 8a and 8b work better than PRIMA-1 (compound 4) in both cancer cell lines.
at 100 µM concentration and most interestingly they have a lower effect on normal cells as compared to the other derivatives. It is intriguing that both derivatives 8a and 8b show a higher effect on cancer cells at a much a lower concentration where PRIMA-1 seems to be ineffective. With these exciting results it was pertinent to study the differential mode of action of derivatives 8a and 8b in NSCLC and normal cells.

Fig. 3: Effect of different derivatives of quinuclidinone on cell viability at a lower concentration. H1299, H1299 p53 +/- and NL-20 cells were treated with 100 µM of different derivatives or left untreated (UC) for 24 hr and then the number of viable cells after treatment was determined using MTT assay. The histogram represents percentage of viable cells (± SD) normalized using untreated control.
1.5 Apoptosis and cell cycle arrest.

Most chemotherapeutics operate by either causing apoptosis or cell cycle arrest or both in conjunction (Hannun, 1997; Hartwell & Kastan, 1994; Houghton, 1999; Kamesaki, 1998). ‘Apoptosis’ was first coined in 1972 by Kerr and described as programmed cell death, differentiating it from necrosis (Kerr, Wyllie, & Currie, 1972). It is a highly regulated process that involves interplay of many molecules present outside and within the cell. This interplay of molecules depends on the stimuli involved, from gamma-radiation, chemotherapeutics, virus, cytokines, UV radiation and many other stresses. Apoptosis is marked by many cellular events that show distinct morphological changes that are characterized by cell shrinkage, nuclear and cytoplasmic blebbing, DNA fragmentation into 200 bp fragments and ultimately the formation of apoptotic bodies that are cleared by phagocytosis. These events are triggered by many biochemical processes that either start from outside the cell or within the cell (Elmore, 2007; Kerr et al., 1972). The main biochemical event that occurs prior to apoptosis becoming irreversible is the activation of effector or executioner caspases 3 and 7 and these are activated through the extrinsic or the intrinsic pathway (Fig. 4) (D. Green & Kroemer, 1998; D. R. Green & Amarante-Mendes, 1998).

1.5.1 Extrinsic apoptosis pathway.

The extrinsic pathway involves the death receptors which are transmembrane cell-surface receptors that have an extracellular domain rich in cysteine residues and an intracellular death domain. The death domain, consisting of 80 amino acids, is essential
in transmitting the extracellular stimuli/stress to initiate an intracellular cascade. These receptors belong to the TNF (tumor necrosis factor) receptor superfamily. The death receptors are mainly activated due to its respective ligand binding. The ligand/receptor interactions have been well studied in apoptosis and they include the FasL/FasR (CD95), TNF/TNFR, Apo-3L/DR3, Apo-2L/DR4 and Apo-2L/DR5. The two main death receptor models in apoptosis are the Fas and TNF receptors (Elmore, 2007).

Fas receptor, upon activation by its ligand, recruits its receptive death domain FADD (Fas associated protein with death domain). Similarly, TNF receptor upon activation by its ligand, recruits TRADD (Tumor necrosis factor receptor-1 associated death domain protein) which then recruits FADD and RIP (receptor interacting protein). After binding of FADD, procaspase 8 also known as FLICE (FADD like interleukin-1-β-converting enzyme) gets recruited through the dimerization of the death effector domain leading to the formation of DISC (death-inducing signaling complex) (Kischkel et al., 1995). This results in the auto catalytic cleavage of procaspase 8 to active caspase 8 thereby initiating the intracellular cascade. Hence, the cleavage of caspase 8 marks the initiation of death receptor apoptosis pathway (Fig. 5) (Martin, Siegel, Zheng, & Lenardo, 1998). Caspase 8 (FLICE) can directly activate caspase 3 by proteolytically cleaving procaspase 3 or it can cleave bid (BH3 interacting domain death agonist protein) to form truncated bid (tBid) that can activate the mitochondrial pathway leading to the cross talk between receptor mediated and intrinsic apoptosis pathways. The main regulator of the extrinsic pathway is c-FLIP (cellular flice like inhibitory protein) that
binds to FADD and caspase 8 and prevents its activity. c-FLIP is similar to caspase 8 but lacks the proteolytic domain (Chang et al., 2002).

Fig. 4: Apoptosis: schematic representation of the extrinsic and intrinsic pathway activators and inhibitors. The extrinsic pathway involves the extracellular signal passing through the death receptors that recruit death domain proteins that mediate the intracellular signal by activating caspase 8 leading to apoptosis. Activation of these receptors also causes the activation of sphingomyelinase that breaks down sphingomyelin to form ceramide. The intrinsic pathway involves the interplay of the bcl-2 related proteins, mainly bcl-2 and bax, where their imbalance causes the release of cytochrome c. Both these pathways activate caspase 3 or 7 that activate enzymes that cause DNA fragmentation. Adapted from the poster entitled ‘Death of a cell – pathways of apoptosis’ with permission of Calbiochem® (Appendix B) (Calbiochem, 2004).
Death receptors can also cause apoptosis by activating important enzymes present in the cellular membrane. These include sphingomyelinase (SMase) enzyme that hydrolyzes sphingomyelin to form ceramide, a known second messenger of apoptosis, and phosphorylcholine. The most common isozymes involved in apoptosis are the neutral sphingomyelinase (NSMase) present in the plasma membrane and acid sphingomyelinase (ASMase) located in the lumen of the lysosome. NSMase is activated by activation of TNF receptor, while TRADD/FADD complex causes the lysosomal ASMase to be activated and transported to the plasma membrane. SMase can be directly activated due to cellular stresses such as UV radiation and chemotherapeutics. Recent reports have shown their presence in the mitochondria and their role in mitochondrial based apoptosis.

Ceramide, the product of this enzymatic reaction, is important in carrying the apoptosis signal into the cytoplasmic fraction and it does so by activating ceramide related kinases that activate stress activated protein kinases (SAPK) or c jun N-terminal kinases (JNKs). These kinases then activate the intrinsic apoptosis pathway. Ceramide can also cause the amplification of the death receptor signal in the absence of its ligand by forming platforms through which these receptors get aggregated and activated causing the recruitment of death domain proteins and activation of caspase 8 (Carpinteiro, Dumitru, Schenck, & Gulbins, 2008; Grassme et al., 2001; Hannun & Obeid, 1995; Hofmann & Dixit, 1998; R. N. Kolesnick & Kronke, 1998; Kurinna, Tsao, Nica, Jiffar, & Ruvolo, 2004; Modrak, Cardillo, Newsome, Goldenberg, & Gold, 2004; Obeid, Linardic, Karolak, & Hannun, 1993).
Fig. 5: Extrinsic apoptosis pathway involving death receptors. The extrinsic pathway is represented by the Fas and TNF receptors that recruit their respective death domain proteins, FADD and TRADD that recruit procaspase 8 forming a complex called DISC. Active caspase 8 is formed by autocatalytic activity leading to apoptosis through activation of caspase 3 or 7. Activation of these receptors also causes the activation of sphingomyelinase that breaks down sphingomyelin to form ceramide, a known second messenger for apoptosis. NSMase, present in the plasma membrane and activated mainly by TNF receptor and ASMase, present in the lysosome, is activated by FADD.
1.5.2 Intrinsic apoptosis pathway.

The intrinsic pathway predominantly involves the non-receptor pathway that triggers the intracellular signaling molecules directly causing apoptosis primarily through the mitochondrial pathway. The mitochondrial membrane potential changes allowing mitochondrial permeability transition pores to form on the outer membrane inducing the release of cytochrome c and SMAC/DIABLO (second mitochondrial-derived activator of caspase/direct IAP binding protein with low pI) from the mitochondria to the cytoplasm (Saelens et al., 2004). Cytochrome c binds to APAF-1 (apoptotic peptidase activating factor-1) forming apoptosome and then recruits procaspase 9 to the complex (Chinnaiyan, 1999; P. Li et al., 1997). This complex causes oligomerization and auto-cleavage of procaspase 9 to active caspase 9 that proteolytically cleaves procaspase 3 to form active caspase 3, the apoptosis effector caspase (Fig. 6). SMAC/DIABLO binds to IAPs (inhibitors of apoptosis) preventing these proteins from suppressing apoptosis by binding to caspases 3, 7 and 9. The mitochondrial pathway is regulated by the bcl-2 related proteins (Deveraux & Reed, 1999; Elmore, 2007; D. Green & Kroemer, 1998; D. R. Green & Amarante-Mendes, 1998; D. R. Green & Reed, 1998; van Loo et al., 2002).
Fig. 6: Intrinsic apoptosis pathway involving the mitochondria and bcl-2 related proteins. The intrinsic pathway involves the change in membrane potential and formation of membrane pores of the mitochondria by the interaction of pro-apoptotic bcl-2 related proteins such as bax and tBid (formed by caspase 8 cleaving bid) causing the release of cytochrome c from the mitochondria to the cytoplasm. Cytochrome c then binds with APAF-1, forming the apoptosome complex. Procaspase 9 is recruited to this complex where it undergoes oligomerization and auto-catalytically cleaves to form active caspase 9 that proteolytically cleaves procaspase 3 to active caspase 3 that triggers apoptosis. The mitochondrial cytochrome c transport is blocked by bcl-2 proteins that form heterodimers with bax.
1.5.3 Bcl-2 related family of proteins involved in apoptosis.

The bcl-2 related proteins are either pro-apoptotic or anti-apoptotic and tightly regulate the mitochondrial pathway. The pro-apoptotic proteins involved in the process are bax (bcl-2 associated X protein), bid, bak (bcl-2 homologous antagonist/killer), bim (bcl-2 like protein 11), bad (bcl-2 associated death promoter protein), bcl-10 (B-cell leukemia/lymphoma 10 protein), bik (bcl-2 interacting killer protein) and blk (B lymphocyte tyrosine kinase) while anti-apoptotic proteins involved are bcl-2 (B cell lymphoma 2), bcl-xL (B cell lymphoma extra large), bcl-xS (B cell lymphoma extra small), bag (bcl-2 associated athanogene) and mcl-1(myeloid cell leukemia sequence 1). Both classes of proteins share common BH3 domains through which they interact with each other. They mainly regulate the release of cytochrome c. The anti-apoptotic proteins block the release of cytochrome c while pro-apoptotic proteins undergo transformational changes that allow these proteins to translocate to the mitochondria causing the formation of pores which changes the mitochondrial potential allowing the release of cytochrome c. The two main proteins, bax and bcl-2, have opposing apoptotic functions within the cell, wherein bax can form homodimers that translocate to the mitochondrial outer membrane causing the release of cytochrome c, while bcl-2 prevents this by forming heterodimers with bax (Gross, Jockel, Wei, & Korsmeyer, 1998). Therefore, the imbalance between these two classes of proteins is essential for determining the fate of apoptosis (Adams & Cory, 1998; Cory & Adams, 2002). A main player that regulates this balance within the cell is p53 through transcription activation of pro-apoptotic genes and suppression of anti-apoptotic genes as mentioned earlier. Both extrinsic and intrinsic apoptosis proteins trigger the activation of apoptosis executioner caspase 3.
1.5.4 Caspases: initiators and executioners of apoptosis.

Caspases are cysteine dependent aspartate specific proteases that are either classified as initiator or executioners of apoptosis. Apoptosis initiator/activator caspases 2, 8, 9 and 10 are involved in initiating the apoptosis signal and carrying it to the executioner caspases. The effectorexecutioner caspases 3, 6 and 7 are key caspases that are activated mostly by initiator caspases, to cause irreversible apoptosis. Caspase 3 activation is a major hallmark of apoptosis. Caspase 3 has many substrates including acinus, lamin, PARP-1 (poly [ADP-ribose] polymerase-1) and CAD/DFF45 (caspase activated deoxyribonuclease/DNA fragmentation factor 45) and each of these substrates causes different cellular events to occur during apoptosis. Lamins are important nuclear and cytoplasmic structural proteins, and when cleaved by caspases they cause chromatin condensation, nuclear membrane breakdown and nucleus and cytoplasm shrinkage, leading to the formation of blebbing and nuclear condensation. CAD/DFF45 is bound in an inactive complex with its inhibitor ICAD during normal conditions. When apoptosis signal is triggered and caspase 3 is activated, it cleaves ICAD and CAD is released to cause DNA fragmentation, another feature seen during apoptosis. Finally, an important feature for apoptosis that is amplified and maintained is the inhibition of repair proteins such as PARP-1. Importantly, caspase 3 causes the cleavage of active PARP-1, which is a 116 kDa protein, to an inactive 86 kDa protein so that the DNA breaks that are formed by CAD are not repaired (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999; Enari et al., 1998; Fischer, Janicke, & Schulze-Osthoff, 2003; Oliver & Vallette, 2005; Pop & Salvesen, 2009; Thornberry & Lazebnik, 1998).
1.5.5 Cell cycle regulation.

Along with apoptosis, cell cycle regulation plays an important role in chemotherapeutic based mode of action. Cells undergo four different phases during a single cell cycle which include the G1, S, G2 and M, wherein the integrity of DNA and chromosome segregation is evaluated before it is passed on to its progeny. S phase is the DNA replication phase and M is the mitosis phase, where the cell undergoes cell division to form daughter cells. Both these phases are energy consuming phases, therefore after each of these phases there is a Gap phase, G1 or G2, where the cell recuperates for the next phase. In G1 phase, the cells assess for any DNA damage before entering the replication phase, while in G2 phase the cells are checked for chromosomal integrity before cell division. When cells encounter DNA damage, repair mechanisms become activated causing cells to arrest at certain cell cycle checkpoints, namely G1/S arrest, S arrest or G2/M arrest, for a short time before entering the next respective phase in the cell cycle. When the damage is irreparable, the cells undergo apoptosis. Many chemotherapeutics cause S phase or G1/S phase arrest mainly through p53 by transcription up-regulation of p21 and Gadd45a proteins (Hartwell & Kastan, 1994; Kohn, Jackman, & O' Connor, 1994; Lee & Bernstein, 1995; Pucci, Kasten, & Giordano, 2000; Smith, Gao, Zhang, Wang, & Dou, 2000).

Quinuclidinone derivatives 8a and 8b mode of action was studied to assess whether these novel potential anti-cancer derivatives cause apoptosis, cell cycle arrest or both in cells on which they show activity and which molecular players are involved in their mechanism.
CHAPTER 2: SIGNIFICANCE AND GOALS

NSCLC is a fast accelerating cancer that is the leading cause of deaths in both males and females in United States and worldwide. It is usually detected in the advanced stage where the only treatment option is chemotherapy (Alberg & Samet, 2003). Chemotherapeutics are cytotoxic agents used to treat cancer; ideally, these are developed to selectively target cancer cells while normal cells are unaffected (Colombo et al., 2001). Unfortunately, this is rare being that many chemotherapeutics do affect normal cells and give rise to a range of side effects. Prolonged treatment with a particular chemotherapeutic causes resistance; therefore, there is growing need to develop effective drugs that have lower side effects and higher activity against cancer especially NSCLC (Chen et al., 2002; Fukuoka, 2000; Marino et al., 1994). For these reasons, there have been several attempts to design small molecules that serve as anticancer drugs specifically triggering apoptosis or reducing proliferation of cancer cells while leaving normal cells unaffected, thereby reducing side effects (Benson et al., 2006; Treskes & van der Vijgh, 1993).

p53, a tumor suppressor protein, controls DNA damage by signaling for cell cycle arrest or apoptosis. Thus, it is not surprising that most chemotherapeutics work better in the presence of p53. Inactivation or mutation of p53 may be the reason for accelerated growth of tumors or resistance to chemotherapeutics. In most cancers, especially lung cancer, p53 is mutated; as, there is much interest in identifying novel compounds that can cause cytotoxicity regardless of p53 status (Fan et al., 1995; Hawkins, Demers, & Galloway, 1996; Vogelstein et al., 2000). Previously, our laboratory reported novel
quinuclidinone analogs that induce cytotoxicity in human non-small cell lung carcinoma (NSCLC) cell line (H1299) null for p53 (Malki et al., 2006). On further preliminary assessment, as seen in Fig. 5, these compounds also affected cells over-expressing p53 suggesting their role in a p53 independent manner. But most importantly these compounds had a lower effect on normal cells. Therefore it is also important to identify the mechanism whereby these analogs induce cytotoxicity for their future use as a chemotherapeutic and in order to develop similar or better compounds.

Apoptosis is induced by most chemotherapeutics and involves interactions between many cellular components including membrane receptors, proteins and lipids, bcl-2 family of proteins and caspases. As discussed earlier, there are many cellular players that cross talk and are involved in apoptosis. In chemotherapeutics that cannot enter the cell, the role of the extrinsic pathway is crucial especially the activation of plasma membrane enzyme sphingomyelinase. Ceramide is a hydrolyzed product of sphingomyelin catalyzed by the SMase. Intracellular ceramide is a second messenger that induces apoptosis in many cellular types during chemotherapy (Lacour et al., 2004; Modrak et al., 2004; Morita et al., 2000). Ceramide may induce apoptosis in lung cancer cells through the JNK pathway. The JNK pathway triggers the mitochondrial pathway by transcriptionally targeting bcl-2 family of proteins (Deng et al., 2001; Kurinna et al., 2004) which in turn help carry the apoptosis signal forward. Two important members of bcl-2 family of proteins are bcl-2 and bax which have opposing functions in controlling apoptosis. The simplest role for bax proposes that bax forms homodimers at the mitochondrial outer membrane altering mitochondrial function and triggering release of
cytochrome c; bcl-2 prevents mitochondrial release of cytochrome c by forming heterodimers with bax thereby preventing the formation of bax homodimers. Therefore, their imbalance can either trigger or block the next signal in the apoptosis progression (Gross et al., 1998; Oltvai, Milliman, & Korsmeyer, 1993; Wolter et al., 1997). Caspases are cysteine proteases that fall into two categories: the initiator caspases 2, 8, 9, 10 and 12 and the effector caspases 3, 6 and 7. Caspase 8 and 9 are the two main initiator caspases activated through the death receptor and the mitochondrial pathway, respectively.

Caspase 3 activation is the key step in nuclear apoptosis where it cleaves important apoptotic inducers, such as DFF45, PARP, acinus and lamin (Fischer et al., 2003; Oliver & Vallette, 2005). Understanding which of these molecular players are involved in triggering 8a and 8b effect against NSCLC and not normal cells will help in developing their use as a therapeutic and future target based therapeutics. These target based drugs will be intended towards specific molecules that either are up-regulated and/or are absent and/or inhibited in cancer cells as compared to normal cells. These developments will help in lowering the side effect profiles seen with most chemotherapeutics and help in improving the quality of life of patients receiving them. Understanding the molecular mechanism will also give insightful perspectives about drug resistance and cancer specific drug choice than the current broad spectrum therapy utilized.

This leads to the goals of the present work that aims at better understanding the molecular mechanisms of quinuclidinone derivatives 8a and 8b that have shown increased anti-cancer potential against NSCLC and lower effect on normal cells.
Goal 1: To investigate the molecular mechanism by which novel quinuclidinone derivatives 8a and 8b cause lower cell viability in cancer cells.

Goal 2: To identify molecular targets involved in the mechanism by which derivatives 8a and 8b show a higher effect on cancer cells and lower effect on normal cells.

Goal 3: To understand the difference in effect of 8a and 8b between cancer cell lines with different p53 status.

Accomplishing these goals will help give a better insight into these two novel compounds that were derived from PRIMA-1 that was effective against cancers with certain p53 point mutations at a very high dose. Both 8a and 8b are effective independent of p53, however, 8a works better in H1299 cells that are null for p53 and 8b works better in H1299 cells over expressing p53. Importantly, both have a lower effect on normal cells. Utilizing the knowledge obtained from 8a and 8b studies will drive drug discovery to develop better acetal derivatives of quinuclidinone that work at even lower concentration and target certain cancer specific molecules.
CHAPTER 3: MATERIALS AND METHODS

3.1 Cell cultures and chemicals

H1299 cells were provided by Dr. Jack Roth, M.D. Anderson Cancer Center. H1299 p53 +/- is a stably transfected cell line having wild type p53 activity. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Hyclone, Utah) supplemented with 10 % (v/v) bovine growth serum (Hyclone) and 1 % (v/v) penicillin streptomycin (Hyclone) and incubated in an environment of 95 % air and 5 % CO2 at 37°C. Normal lung epithelial cell line (NL-20, obtained from ATCC, Virginia) was cultured in DMEM/F-12 media (Gibco, California) supplemented with 10 % FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, insulin (1.2 g/L), transferrin (0.001 mg/mL), EGF (20ng/μL) and hydrocortisone (500 ng/mL) at 37 °C in a 5 % CO2 atmosphere.

All quinuclidinone derivatives for the study were provided by Dr. Stephen Bergmeier’s laboratory, Ohio University, Athens, OH. Both 8a and 8b affect cell viability in a dose dependent manner as seen from previous study (Malki et al., 2006). For the current study treatment, quinuclidinone derivatives were prepared in 100 μM concentration using appropriate media. 100 μM was the initial concentration used for all the quinuclidinone derivatives prepared to study decrease in cell survival percentage in H1299, H1299 p53 +/- and NL-20 cells. Previous studies indicated 100 μM was the concentration at which 8a and 8b were most effective in the cancer cells and had a lower effect in normal cells as compared to other derivatives at the same dose; therefore, 100 μM was used in the current study to maintain consistency in results.
Inhibitors used were GW4869 (15 µM), desipramine (10 µM) and fumonisin B1 (50 µM) purchased from Sigma (St. Louis, MO, USA).

3.2 Qualitative measurement of apoptosis using TUNEL assay along with DAPI staining

TUNEL (Terminal deoxynucleotidyl tranferase dUTP nick end labeling) assay was employed for in-situ detection of apoptosis using DeadEnd™ fluorimetric tunel system (Promega, USA). Cells were cultured on 4-chamber slides (VWR, USA) at a density of $2 \times 10^4$ cells per chamber for 24 hr and then treated with 100 µM of the derivatives for 24 hr. Treated cells were washed with cold PBS and fixed with 4 % paraformaldehyde (PFA) for 20 min at 4 °C. The fixed cells were incubated with digoxigenin-conjugated dUTP in terminal deoxynucleotide transferase recombinant (rTdT)-catalyzed reaction and nucleotide mixture for 60 min at 37 °C in a humidified atmosphere after which the slides were immersed in stop/wash buffer for 15 min at room temperature. Finally, the cells were washed with PBS to remove any unincorporated fluorescein-12-dUTP. For nuclei staining the cells were incubated in DAPI solution (1 µg/ml) for 15 min in the dark. The slides were observed under immunofluorescence microscope (ECLIPSE E600, Nikon,; West Chester, OH) and images were taken using digital camera (SPOT, DC Imaging) at 100x magnification. The images were merged using the Advanced SPOT software (Diagnostic Instrument).
3.3 Quantitative Measurement of Apoptosis using Cell Death Detection ELISA assay

Cells were seeded at a density of 2 X 10⁴ per well in a 96-well plate and incubated for 24 hours. Different analogs were added by changing culture media with media containing derivatives and incubated for an additional 24 hr. ELISA assay was performed using a Cell Death Detection ELISAPLUS kit (Roche-Applied Science, Indianapolis, USA) that measures mono or oligo nucleosomes (DNA-Histone complex) released from fragmented DNA in apoptosing cells. Briefly, cells were lysed with 200 μL lysis buffer for 30 min at room temperature. The lysate was centrifuged at 200 g for 10 min. 150 μL of supernatant was collected, of which 20 μL was transferred to a streptavidin-coated microplate and then incubated with anti-histone biotin labeled and anti-DNA peroxidase labeled antibodies at room temperature for 2 hr. After washing with incubation buffer three times, 100 μL of substrate solution (2,2’azino-di(3-ethylbenzthiazolin-sulphuric acid) was added to each well and incubated for 15-20 min at room temperature. The absorbance was measured using an ELISA reader (Spectra Max Plus) at 405 nm. Each assay was performed in triplicate and standard deviation determined.

3.4 Caspase-3 activity determination

Caspase-3 enzyme activity was measured using EnzChek® Caspase-3 assay kit 1 (Molecular Probes, Invitrogen, USA). The cells were treated with 100 μM 8a and 8b for 24 hr and assayed according to the manufacturer’s protocol. Cells were lysed using lysis buffer provided in the kit. Supernatant collected after centrifugation was used for the assay as well as for protein estimation as described latter. The supernatant was mixed
with substrate Z-DEVD-AMC in a 96-well plate. Control without enzyme was assayed for each experiment to determine the background fluorescence. Caspase-3 inhibitor (Ac-DEVD-CHO inhibitor) provided in the kit was added to control wells. Fluorescence was measured at excitation wavelength of 342 nm and emission wavelength of 441 nm. Each experiment was performed in triplicate and statistical significance determined.

3.5 Cell cycle analysis using flow cytometry

Cells were seeded at a density of 3-5 X 10^5 in 100 mm plates. Cells were then switched to serum-free media to synchronize the cell cycle. After 24 hr, the media was changed to complete media containing 8a or 8b (100 µM). Cells were harvested after 24 hr by trypsinization and washed with cold PBS and fixed with ice-cold 70 % ethanol overnight. Finally, the cells were centrifuged and cell pellets were resuspended in PBS containing 5 µg/mL RNase A (Sigma, St. Louis, MO) and 50 µg/mL propidium iodide (Sigma) for analysis. Cell cycle analysis was performed using FACScan Flow Cytometer (Becton Dickinson) according to the manufacturer’s protocol. Modfit software was used to calculate the cell-cycle phase distribution from the resultant DNA histogram, and expressed as a percentage of cells in the G1 (G0/G1), S and G2 (G2/M) phases. Each assay was performed in triplicate and standard deviation determined.

3.6 Analysis of sphingomyelinase activity using Amplex Red sphingomyelinase assay

Sphingomyelinase activity was determined in vitro by Amplex Red sphingomyelinase assay (Molecular Probes™, Invitrogen, USA) in a 96-well microplate
reader according to manufacturer’s protocol. Briefly, 4000 - 5000 cells/well in 100 µL of medium were seeded in a 96-well plate for 24 hr prior to drug treatment. The media was then changed to media with analogs (100 µM). Samples were diluted with reaction buffer and 100 µL was pipetted into a new 96-well microplate. 100 µL of 100 µM Amplex Red reagent containing 2 U/ml horseradish peroxidase (HPR), 0.2 U/ml choline oxidase, 8 U/ml alkaline phosphatase and 0.5 mM sphingomyelin working solution was added to each sample and incubated for 30 min and protected from light. Fluorescence was measured in a fluorescence microplate reader (Spectra Max Plus) using excitation range of 530-560 nm and emission detection at 590 nm. Each experiment was performed in triplicate and standard deviation determined.

3.7 FACS analysis for ceramide measurement

Cells treated with or without derivatives 8a and 8b (100µM) were fixed with formaldehyde (final concentration 4 % in PBS) for 10 min at 37 ºC and then chilled on ice for 1 min. Cells were then permeabilized using ice-cold 90 % methanol for 30 min on ice. Permeabilized cells were then washed and resuspended in PBS and placed in a round bottomed 96-well plate. They were further incubated for 45 minutes with mouse monoclonal anti-CER 15B4 (Alexis, Coger, Paris, France). Cells were then washed with PBS containing 0.5 % heat inactivated FBS and incubated for another 30 min with (FITC)-labeled goat anti-mouse (BD Biosciences, USA). The cells were washed for a final time with PBS and then subjected to fluorescent activated cell sorter (FACS) analysis (Becton Dickinson) and data was analyzed using CellQuest software.
3.8 Ceramide measurement using dot blot assay

Cell pellets of untreated and treated samples were collected and lysed on ice for 30 min using lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1 % triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 10 % glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors). To remove cell debris, the extracts were centrifuged at 13,000 rpm for 15 min at 4 °C. To detect ceramide level in the treated and untreated cells, the centrifuged extracts were applied to PVDF membrane placed in a Dot Blot apparatus (Biorad, USA). Protein concentration was first determined using Folin-Lowry protein assay and equivalent concentrations were then spotted. Ceramide level was analyzed using primary anti-ceramide antibody (Sigma, St. Louis, MO) and then probed using secondary antibody conjugated to HRP. Protein was visualized using ECL chemiluminescence kit (GE, USA).

3.9 Cell viability analysis using MTT assay for inhibitor analysis

Cell viability was measured using the methyl tetrazolium (MTT) bromide mitochondrial activity assay (ATCC, Manassas, VA USA) according to the manufacturer’s protocol. Cells (at a density of 5-10 X 10^3 per well) in 100 µL of medium were added to a 96-well plate and incubated for 24 hr. The media was then changed to media containing inhibitors and/or 100 µM of drugs. After 24 hr incubation, 10 µL of MTT reagent was added to each well and incubated for 4 hr at 37 °C. After incubation, 100 µL of detergent was added to dissolve the formazan crystals and incubated overnight
at RT in the dark. The absorbance was then measured at 570 nm. Each assay was performed in triplicate and statistical analysis performed.

3.10 Pro-apoptotic and anti-apoptotic protein determination using western blot analysis

Total protein was extracted from treated and untreated cells using lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1 % triton X-100, 150 mM NaCl, 1 mM dithiothretol, 10 % glycerol, 0.2 mM phenylmethylsulphonyl fluoride and protease inhibitors) for 30-50 min on ice. The extracts were centrifuged at 13,000 rpm for 15 min at 4 °C to remove cell debris. Folin Lowry (Pierce, USA) protein assay was used to determine the protein concentration in the cell lysates. Proteins were resolved by electrophoresis on 8-12 % sodium dodecyl sulphate – polycrylamide gels. The resolved proteins were transferred onto PVDF membrane and then probed with primary antibody against the protein of interest prepared in 5 % milk/PBS-T. The membrane was washed using PBS-T and then appropriate secondary antibody conjugated to HRP was used for visualization of the bands using ECL chemiluminescence kit (GE, USA). Anti-caspase 3 (1:200 dilution), anti-PARP-1 (1:200 dilution was used), anti-caspase 8 (1:200 dilution), anti-JNK-1P (1:200 dilution), anti-p53 (1:800 dilution), anti-bax (1:200 dilution), anti-bcl-2 (1:200 dilution), anti-caspase 9 (1:200 dilution) and anti-GAPDH (1:200 dilution) antibodies were purchased from Santa Cruz Biotechnology ®, Inc (CA, USA). Anti-β-actin (1:10,000 dilution) antibody was purchased from Sigma (St. Louis, MO, USA). Pixel density of the proteins studied was calculated using Image J, version 1.41o, NIH.
The values obtained were first normalized to loading control (GAPDH/β-actin) and fold increase was measured by comparing to the untreated control (0 hr) value. At least two or more independent experiments were performed.

### 3.11 Changes in p53 localization using immunofluorescence:

Cells as indicated were grown on cover slips in 35 mm dish 24 hr prior to treating with 100 μM quinuclidinone 8b. After 24 hr treatment the growth media was removed and the cells were washed with 1X PBS then fixed with 95 % ethanol and 5 % acetic acid at 20 ºC for 5 min. The cells were washed with PBS and blocked with 2 % chicken serum prepared in PBS for 15 min. The cells were incubated with primary antibody Bp53-12 (Santa Cruz Biotechnology ®, Inc, CA, USA) prepared at 1:400 dilution in 2 % chicken serum/PBS for 1 hr at RT. After incubation the cells were washed and incubated in the dark with FITC-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology ®, Inc, CA, USA) prepared at 1:100 dilution in 2 % chicken serum/ PBS for 30 min. The cells were washed with PBS and mounted on glass slides using a drop of Vectashield with DAPI (Vector Lab Inc; Burlingame, CA). The slides were observed under immunofluorescence microscope (ECLIPSE E600, Nikon; West Chester, OH) and images were taken using digital camera (SPOT, DC Imaging) at 400x magnification. The images were merged using the Advanced SPOT software (Diagnostic Instrument).
3.12 Bax promoter activity using Dual Luciferase assay:

Cells were transfected using lipofectamine 2000 (Invitrogen; Carlsbad, CA) with Bax luciferase reporter plasmid construct (Panomics, Fremont, CA), a firefly luciferase reporter plasmid constructed with bax promoter sequence. pRL-TK vector (Promega; Madison, WI) and Renilla luciferase HSV-thymidine kinase promoter plasmid was co-transfected to provide constitutive expression of the Renilla luciferase as an internal control. After 4 hr, the transfection media was removed and fresh new growth media was added for 8 hr. After the incubation the culture media was changed to media containing/not containing 100 µM of quinuclidinone 8a or 8b. After 24 hr of treatment the expression of luciferase was measured using the dual luciferase reporter assay kit (Promega, Madison, WI). Following the manufacturer’s protocol, the treated and untreated cells were lysed and 20 µl of the supernatant was used for analysis. The relative luminescence intensity was measured using a luminometer (Lumat LB9507, BERTHOLD Technologies; Oak Ridge, TN). The bax promoter activity was normalized using Renilla luciferase activity. Each experiment was conducted in triplicate and statistical analysis performed.

3.13 Bax transcript level determination using Real-Time RT PCR:

Total RNA was extracted using RNeasy total RNA extraction kit (Qiagen, Inc.; Valencia, CA) according to the manufacturer’s protocol from H1299 and H1299 p53 +/- cells treated with 100 µM of quinuclidinone derivatives 8a/8b or untreated control. Total extracted RNA (1-3 µg) was reverse transcribed using Bio-Rad iScript™ Select cDNA
Synthesis kit (Bio-Rad; Hercules, CA, USA) in the Effendorf Mastercycler gradient PCR machine (Effendorf; Westbury, NY, USA). Real Time PCR master mix for bax was prepared according to the manufacturer’s protocol with RT² PCR primer set for human bax (SuperArray; Frederick, MD, USA) (12.5 µL of Bio-Rad iQ™ SyBr Green Supermix, 1 µL of cDNA, 1 µL of RT² PCR primer set bringing the total volume to 25 µL with water); the human β-actin (Biosynthesis; Lewisville, TX, USA) master mix was prepared similarly except the primer concentration used was 10 µM. Bio-Rad iCycler (Bio-Rad; Hercules, CA, USA) was used to run PCR reaction and ΔΔCt method was used for data analysis. Each experiment was conducted in triplicate and statistical analysis performed.

3.14 Mitochondria and cytosol protein isolation using differential centrifugation for detection of cytochrome c:

H1299 and H1299 p53 +/- cells were treated with 100 µM quinuclidinone derivative 8a and 8b or left untreated for 24 hr. After treatment the cells were washed with ice-cold 1X PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and resuspended in 1 mL isolation buffer pH 7.5 (250 mM sucrose, 10 mM HEPES, 1mM EDTA, 10 µl of proteinase inhibitors cocktail and 1 mM PMSF). After 20 mins incubation on ice, the suspensions were dounced (3-4 times), checked for lysis with trypan blue then the nuclei and unbroken cells were pelleted by centrifugation at 500 g for 10 min at 4 ºC. The supernatants were re-centrifuged at 500 g for 10 min at 4 ºC to remove any residual nuclei or unbroken cells. The supernatants were then centrifuged at
7900 g for 10 min at 4 °C to pellet the mitochondria which were resuspended in 200 µl isolation buffer and the resultant supernatant was designated as the cytosolic fraction. 50 µg of both the mitochondrial and cytosolic proteins were then western blotted for anti-cytochrome c (1:200 dilution) antibody from Santa Cruz Biotechnology ®, Inc (CA, USA).

3.15 Apoptosis gene array analysis using Real-Time RT PCR:

Treated and untreated cells were subjected to total RNA extraction using RNeasy total RNA extraction kit (Qiagen, Inc.; Valencia, CA, USA). Before preparing the cDNA, the quality of RNA isolated was evaluated using Human RT² RNA QC PCR Array (SABiosciences; Frederick, MD, USA) then reverse transcription polymerase chain reaction (RT-PCR) was performed on 1 µg of respective total RNA using RT² First Strand Kit (SABiosciences; Frederick, MD, USA). The cDNA were mixed with RT² qPCR Master Mixes (SABiosciences; Frederick, MD, USA) according to the manufacturer’s protocol and then aliquoted to the Human Apoptosis RT² Profiler™ PCR Array (SABiosciences; Frederick, MD, USA) containing 84 relevant apoptosis genes and 5 housekeeping genes (Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, Hypoxanthine phosphoribosyltransferase 1, Ribosomal protein L13a, β-2-microglobulin) primer sets, RNA transcription controls, genomic DNA control and positive PCR controls on a 96-well plate. The PCR reaction was performed in Bio-Rad iCycler (Bio-Rad; Hercules, CA) and fold change in expression levels of the genes under study was
calculated using ΔΔCt method. Ribosomal protein L13a, GAPDH and β-actin were used as internal control for normalization.

3.16 Statistical analysis:

All data were analyzed by student-t test and expressed as mean ± standard deviation. A p-value of ≤ 0.05 was considered statistically significant.
CHAPTER 4: RESULTS

4.1 Novel quinuclidinone 8a and 8b affect cell growth of lung cancer cells by inducing apoptosis and S phase arrest

To investigate the mechanism by which the quinuclidinone derivatives cause cell growth inhibition, two human epithelial NSCLC cell lines (H1299 and H1299 p53 +/-) and human normal lung epithelial cell line (NL-20) were used in keeping with the earlier investigations. Both 8a and 8b induce apoptosis as studied using TUNEL assay, Cell Death Detection assay, caspase 3 activity and processing of PARP-1 (Figs. 7-10) and S phase arrest as demonstrated using propidium iodide (PI) staining (Fig. 11).

4.1.1 Novel quinuclidinone derivatives 8a and 8b induce apoptosis.

Most chemotherapeutics induce apoptosis as one of their principle modes of action (Hannun, 1997; Houghton, 1999). In H1299 and H1299 p53 +/- cells, 8a and 8b at 100 µM concentration after 24 hr, induced apoptosis as measured using TUNEL assay (Fig. 7). NL-20 treated cells also showed apoptosis positive cells but to a lesser extent as compared to H1299 and H1299 p53 +/- treated cells (Fig. 7).

4.1.2 Treatment with 8a and 8b increased more apoptosis of NSCLC (H1299 and H1299 p53 +/-) cells than normal lung (NL-20) cells.

To quantify the extent of apoptosis induced by 8a and 8b between these cell lines as compared to other derivatives, Cell Death Detection assay was employed. Both 8a and 8b showed an approximate 4-fold increase in apoptosis as compared to the untreated
control in H1299 cells (Fig. 8). In H1299 p53 +/+ treated cells a similar trend was observed, wherein > 3-fold increase in apoptosis was detected (Fig. 8). In contrast to the other derivatives, 8a and 8b had lower soluble nucleosome formation in NL-20 cells as compared to the cancer cells (Fig. 8).

**Fig. 7: Novel quinuclidinone derivatives 8a and 8b induce apoptosis.** Human non-small lung epithelial (H1299 and H1299 p53 +/-) and normal lung epithelial (NL-20) cells (2 X 10^4) were treated with or without (UC) 100 µM of derivatives 8a and 8b for 24 hr. Analysis was done by fluorescent microscopy using fluorescein-dUTP (FITC panel) for DNA fragments and nuclei were stained with DAPI. Photographs were taken at 100x magnification. Results are representative of 3 independent experiments.
Fig. 8: Treatment with 8a and 8b increased more apoptosis of NSCLC (H1299 and H1299 p53 +/+ ) cells than normal lung (NL-20) cells. H1299 and H1299 p53 +/+ and NL-20 cells were treated with 100 µM of different derivatives or left untreated (Control) for 24 hr and analyzed for mono- or oligo-nucleosomes released after DNA fragmentation as described in Materials and Methods. The histogram represents mean absorbance (± SD) measured at 405 nm of 3 independent experiments done in triplicate. Significance as compared to untreated control was * p < 0.05.

4.1.3 Up-regulation of caspase 3 activity by novel quinuclidinone derivatives 8a and 8b.

As discussed earlier in section 1.5, caspase 3 is a known effector of apoptosis and in its active form functions by cleaving DFF45 (DNA fragmentation factor 45), PARP (poly ADP- ribose polymerase), acinus and lamin. Here, the activity of this enzyme in
H1299 and H1299 p53 +/+ cells treated with 100 µM of 8a and 8b was evaluated. These experiments showed that 8a induced caspase 3 activity more significantly ($p \leq 0.01$) than 8b ($p \leq 0.05$) in H1299 cells as compared to its untreated control (Fig. 9A). Similarly, 8b significantly ($p \leq 0.05$) increased the enzyme activity in H1299 p53 +/+ cells as compared to the untreated control (Fig. 9B). Furthermore, procaspase-3 (32KD full length fragment) expression determined using western blot analysis decreased 24 hr after treatment with 8a and 8b in H1299 and H1299 p53 +/+ cell lines which correlates with the activation of caspase-3 enzyme (Fig. 9C).
Fig. 9: Up-regulation of caspase 3 activity by novel quinuclidinone derivatives 8a and 8b. H1299 (A/C) and H1299 p53 +/+ (B/C) cells were treated with 100 µM of 8a and 8b or left untreated (Untreated Control, UC) for 24 hr. Endogenous caspase 3 activity was determined for H1299 (A) and H1299 p53 +/- (B) cells as described in Materials and Methods and Ac-DEVD-CHO inhibitor was used to indicate the background fluorescence. The histogram represents mean fluorescence (± SD) measured at excitation/emission 496/520 nm of 3 independent experiments done in triplicate. Significance as compared to the untreated control was * p < 0.05 and ** p < 0.01. (C) Procaspase 3 levels were determined by western blot analysis using mouse anti-caspase 3 antibody from whole cell lysates and β-actin was used as the loading control. Results are representative of 3 independent experiments.
4.1.4 Time course PARP-1 processing by 8a and 8b treatment.

PARP-1 repairs DNA damage, but when cleaved from 116 kDa to 89 kDa by serine protease caspase-3, the activity decreases (Soldani & Scovassi, 2002). 8a induced time dependent processing of PARP-1 in H1299 cells; as the treatment time increased there was decrease in the 116 kDa active form and the inactive cleaved 89 kDa protein increased. While in H1299 p53 +/+ cells treated with 8a, the 116 kDa active form decreased in a time dependent manner but the 89 kDa inactive form did not increased until 6 hr of treatment (Fig. 10A). Similarly, 8b induced time dependent processing of PARP-1 in H1299 cells wherein the 116 kDa active form decreased and the 89 kDa protein inactive form increased with time. However, in H1299 p53 +/+ cells there was decrease in the 116 kDa active form at 6 and 24 hr treatment with a transient increase at 12 hr but a time dependent continuous increase in the 89 kDa inactive form (Fig. 10B). However, both 8a and 8b induced maximum processing after 24 hr treatment as compared to the untreated control (0 hr) in H1299 and H1299 p53 +/+ cells correlating with the caspase 3 activity.

(A) | H1299 cells | H1299 p53 +/+ cells |
--- | --- | --- |
8a | 0 1 6 12 24 | 0 1 6 12 24 (hr) |
PARP-1 (116 kDa) | | |
PARP-1 (89 kDa) | | |
GAPDH | | |
Fig. 10: Time course PARP-1 processing by 8a and 8b treatment. H1299 and H1299 p53 +/- cells were treated with 100 µM of 8a (A) and 8b (B) for indicated time intervals. PARP-1 cleavage was determined by western blot analysis using mouse anti-PARP-1 antibody from whole cell lysates that detects the 116 kDa full-length and 89 kDa cleavage product. GAPDH was used as the loading control. Results are representative of 3 independent experiments.

4.1.5 Novel quinuclidinone derivatives 8a and 8b induce S phase arrest.

To determine if apoptosis stimulation was attributed to the changes in cell cycle regulation, the influence of 8a and 8b on cell cycle distribution was performed with propidium iodide staining. The experiments revealed that 8a induced significant (p < 0.05) accumulation of cells in S phase with decrease in G0/G1 and G2/M phase in H1299 cells (Fig. 11A/B). While in both cell lines 8b treatment demonstrated a more significant (p < 0.01, p < 0.001) accumulation of cells in S phase as compared to the untreated control (Fig. 11A/B).
Fig. 11: Novel quinuclidinone derivatives 8a and 8b induce S phase arrest. H1299 (A) and H1299 p53 +/- (B) cells were treated with 100 μM of 8a and 8b or left untreated (Untreated Control) for 24 hr and analyzed by flow cytometry as described in Materials and Methods. The histogram represents mean percentage of cells (± SD) in different phases as analyzed using Modfit software of 3 independent experiments. Significance as compared to the untreated control was * p < 0.05, ** p < 0.01 and *** p < 0.001.
4.2 Novel quinuclidinone 8a and 8b induce sphingomyelinase mediated apoptosis.

To further understand the molecular mechanism by which the quinuclidinone derivatives exert better affect on cancer cells than normal cells, the apoptosis signaling cascade was investigated. Both 8a and 8b induce Smase activity with an up-regulation in its downstream targets ceramide, JNK-1 and caspase 8 in H1299 and H1299 p53 +/- cells (Figs. 12-17).

4.2.1 Up-regulation of SMase activity by 8a and 8b treatment in H1299 and H1299 p53 +/- compared to NL-20 cells.

SMase has been implicated in apoptosis of cells treated with anti-tumor agents. The activity of the enzyme in H1299, H1299 p53 +/- and NL-20 cells treated with 8a and 8b was evaluated. The experiment revealed that 8a and 8b induced an increased sphingomyelinase activity in H1299 and H1299 p53 +/- cells as compared to their untreated counterparts and to the treated NL-20 cells (Fig. 12). Notably, 8a treated H1299 cells have a significant (p < 0.05) increase in SMase activity and 8b has more prominent effect in H1299 p53+/+ cells (Fig. 12).
Fig. 12: Up-regulation of SMase activity by 8a and 8b treatment has a greater effect in H1299 and H1299 p53 +/- than NL-20 cells. H1299, H1299 p53 +/- and NL-20 cells were treated with 100 µM of different derivatives or left untreated (Cells only) for 24 hr. Endogenous SMase activity was determined as described in Materials and Methods. The histogram represents mean fluorescence (± SD) measured at 560/590 nm (excitation/emission) of 3 independent experiments. Significance as compared to the untreated control was * p ≤ 0.05.
4.2.2 Effect of inhibiting sphingomyelinase on cell viability after treatment with 8a or 8b.

To determine whether these changes in sphingomyelinase activity plays a role in the ability of 8a and 8b to induce cytotoxicity in H1299 and H1299 p53 +/- cells, specific inhibitors of neutral sphingomyelinase (GW4869 15 µM) and acid sphingomyelinase (Desipramine 10 µM) were used. Smase activity assays were performed to verify that the concentration of inhibitor used inhibited the induction of SMase by 8a and 8b treatment (Fig. 13A). In H1299 cells treated with 8a, both inhibitors reduced the enzyme activity by more than 90 % while in 8b treated cells, neutral SMase inhibitor had a better effect in reducing the enzyme activity by more than 80 % but acid inhibitor reduced the activity by less than 40 % (Fig. 13A). In cells over-expressing p53, desipramine, the acid SMase inhibitor, reduced > 70 % of the enzyme activity in 8a treated cells and in 8b treated cells neutral SMase inhibitor had a similar reduction of more than 60 % of the activity (Fig. 13A).

In keeping with the SMase inhibitor activity analysis, the cell viability experiments revealed that in H1299 cells treated with 8a, both inhibitors altered the cell viability of treated cells but a more significant effect (p ≤ 0.01) was observed with the neutral SMase inhibitor treated cells. In 8b treated H1299 cells, GW4869 significantly abrogated the ability of 8b to reduce cell viability but desipramine had no effect as compared to the 8b treated cells with no inhibitor (Fig. 13B). In H1299 p53 +/- cells treated with 8a, desipramine had a significantly (p ≤ 0.05) better effect in inhibiting 8a anti-cancer effect. While in 8b treated H1299 p53 +/- cells, GW4869 significantly (p ≤
0.05) abrogated 8b’s anti-cancer effect (Fig. 13B). These events suggest that SMase was one of the possible molecular targets of 8a and 8b and for this reason we examined the effect of 8a and 8b on ceramide generation.
Fig. 13: Effect of inhibiting sphingomyelinase on cell viability after treatment with 8a or 8b. H1299 and H1299 p53 +/+ cells were treated with or without (No Smase Inhibitor), Neutral Smase (GW4869 15 µM), or Acid Smase (Desipramine 10 µM) inhibitors for 1 hr before treating the cells with 100 µM 8a or 8b or left untreated (UC/C) for 24 hr. (A) Cell were analyzed for SMase activity as described in Materials and Methods. The histogram represents mean fluorescence (± SD) measured at 560/590 nm (excitation/emission) of triplicate measurement. (B) The number of viable cells after treatment was determined using MTT assay as described in Materials and Methods. The histogram represents percentage of viable cells (± SD) normalized using untreated control. The results are indicative of 3 independent experiments. Significance as compared to the treatment without inhibitor was * p ≤ 0.05 and ** p ≤ 0.01.
4.2.3 Effect of 8a and 8b on endogenous ceramide production.

Neutral and acid sphingomyelinase are involved in endogenous ceramide production, a known second messenger for apoptosis (Hannun & Obeid, 1995). Thus, sphingomyelinase activation in cancer cells was further assessed by determining the intracellular level of ceramide. In keeping with the sphingomyelinase activation trend, 8a induced significantly more ceramide generation in H1299 cells after 24 hr treatment as compared to the untreated control (p ≤ 0.01). 8b induced ceramide significantly in H1299 p53 +/+ cells as compared to the untreated control (p ≤ 0.05) (Fig. 14A/B).
Fig. 14: Effect of 8a and 8b on endogenous ceramide production. H1299 and H1299 p53 +/+ cells were treated with or without (Untreated Control) 100 µM 8a or 8b for 24 hr and assayed for ceramide production using (A) Dot blot assay with mouse monoclonal anti-ceramide antibody and (B) by flow cytometry by using FITC-labeled mouse monoclonal anti-CER15B4. The histogram represents mean fluorescence (± SD) subtracting Isotype control fluorescence of 3 independent experiments done in triplicate. Significance as compared to untreated control was * p < 0.05 and ** p < 0.01.

4.2.4 Effect of de novo ceramide production inhibitor on 8a or 8b treatment.

To evaluate whether the ceramide generated is due to de novo synthesis, H1299 and H1299 p53 +/+ cells were treated with/without 50 µM fumonisin B1, a potent ceramide synthase inhibitor and with/without 100 µM of 8a and 8b or left untreated for
The cell viability assays with fumonisin B1 inhibitor revealed that they followed a similar trend to cells treated with 8a or 8b alone (Fig. 15).

**Fig. 15: Effect of de novo ceramide production inhibitor on 8a or 8b treatment.**

H1299 and H1299 p53 +/- cells were treated with ceramide synthase inhibitor (Fumonisin B1 50 µM) or without (No Inhibitor) along with or without (C) 100 µM 8a or 8b for 24 hr and analyzed for cell viability using MTT assay. The histogram represents percentage of viable cells (± SD) normalized using untreated control. The results are indicative of 3 independent experiments done in triplicate. Significance as compared treatment without inhibitor was * p < 0.05.
4.2.5 Time course up-regulation of JNK phosphorylation by 8a and 8b.

Previous studies have documented that ceramide induces apoptosis through the phosphorylation of stress activated enzyme/ c-Jun NH₂-terminal kinase (JNK) in lung cancer cells (Kurinna et al., 2004). Experiments were conducted to investigate whether 8a and 8b induced phosphorylation of JNK. Total JNK protein expression increased after 24 hr treatment (Fig. 16A) and phosphorylated JNK was detected in both cell lines 1 hr after treatment and gradually increased through 24 hr as compared to the untreated control (Fig. 16B/C). With 8a treatment, the 54 kDa isoform was mostly phosphorylated (Fig. 16B) while with 8b treatment, both 54 and 46 kDa isoforms were phosphorylated (Fig. 16C).
Fig. 16: Time course up-regulation of JNK phosphorylation by 8a and 8b. H1299 and H1299 p53 +/+ cells were treated with or without (UC) 100 µM 8a and 8b for 24 hr. (A) Total JNK level was determined from whole cell lysates by western blot analysis using monoclonal antibody that detects JNK p46 and p54 isoforms. β-actin was used as the loading control. H1299 and H1299 p53 +/+ cells were treated with 100 µM 8a (B) and 8b (C) for the indicated time intervals and the cell lysate was subjected to western blot analysis to determine level of phosphorylated JNK using anti-p-JNK-1/p38 antibody detecting Thr 183 and Tyr 185. GAPDH was used as the loading control. Results are representative of 3 independent experiments.
4.2.6 8a and 8b induce procaspase 8 processing in a time dependent manner.

Both neutral and acid sphingomyelinase enzymes are activated by death receptors (Adam-Klages et al., 1998) and procaspase 8 is cleaved on activation of death receptors to form activated caspase 8 (Martin et al., 1998). Therefore to examine whether death receptors are involved in 8a and 8b induced cell death, processing of procaspase 8 protein was determined using western blot analysis at different time intervals after treatment with the derivatives 8a or 8b. 8a induced the processing of procaspase 8 as early as 1 hr after treatment and significantly by 6 hr after treatment in H1299 cells (Fig. 17A). 8b induction of procaspase 8 processing was similar for both cell lines (Fig. 17B). Altogether, these results suggested that in H1299 and H1299 p53 +/- cells, 8a and 8b induced sphingomyelinase through the death receptors with production of ceramide and phosphorylation of JNK. Moreover, it appeared that 8a and 8b effect was strongest at 24 hr after treatment where apoptosis was detected.
Fig. 17: 8a and 8b induce procaspase 8 processing in a time dependent manner.

H1299 and H1299 p53 +/- cells were treated with 100 µM 8a (A) and 8b (B) for the indicated time intervals and the cell lysate was subjected to western blot analysis to determine procaspase 8 processing using anti-caspase 8 antibody that detects the 55 kDa precursor of caspase 8. The numbers between the procaspase 8 and GAPDH panels indicate fold change in pixel density values of procaspase 8 as compared to its untreated control and normalized to its respective GAPDH pixel density value. Results are representative of 3 independent experiments.
4.3 8a and 8b induce the intrinsic mitochondrial pathway.

Earlier studies demonstrated activation of caspase 8 and JNK (Figs. 10-11) and its known that both caspase 8 and JNK can activate the mitochondrial pathway for apoptosis through key pro-apoptosis proteins, bax and caspase 9. 8a and 8b induced bax expression with decrease in bcl-2 expression, release of cytochrome c to the cytoplasm from the mitochondria, and increased caspase 9 expression as demonstrated by western blot analysis (Figs. 18-21).

4.3.1 Novel Quinuclidinone derivatives 8a and 8b induce bax protein expression.

Bax, an important member of the bcl-2 family of proteins, regulates intrinsic apoptosis by triggering cytochrome c release from the mitochondria to induce apoptosis (Gross et al., 1998). Therefore, changes in bax protein expression level in H1299 and H1299 p53 +/- cells treated with 8a or 8b by western blot analysis were analyzed. Pixel density values show that in H1299 cells, 8a induced maximum expression of bax protein after 24 hr treatment. In H1299 p53 +/- cells, bax protein level increased notably after 6hr of treatment (Fig. 18A). While in H1299 cells treated with 8b, increase in bax protein expression was transient and started to decrease after 24 hr treatment while in H1299 p53 +/- cells 8b induced increase in bax expression after 1 hr of treatment and notably after 6 hr of treatment (Fig. 18B).
Fig. 18: Novel Quinuclidinone derivatives 8a and 8b induce bax protein expression.

H1299 and H1299 p53 +/- cells were treated with 100 µM 8a (A) or 8b (B) for the indicated time interval and bax protein expression level was determined from whole cell lysates by western blot analysis using mouse monoclonal anti-Bax antibody. GAPDH was used as the loading control. The numbers between the bax and GAPDH panels indicate fold change in pixel density values of bax as compared to its untreated control and normalized to its respective GAPDH pixel density value. Results are representative of 3 independent experiments.
4.3.2 Down-regulation of Bcl-2 protein by 8a and 8b.

Bcl-2, also a member of Bcl-2 family of proteins, has opposing function to bax in regulating intrinsic mitochondrial pathway wherein it blocks bax activity by forming heterodimers with it and preventing the cytochrome c release (Tan et al., 2006). Therefore, to gain insight on 8a and 8b effect on bcl-2 protein levels in both cell types, western blot analysis was used. In both cell lines, 8a and 8b down-regulated bcl-2 protein expression as compared to untreated control (Fig. 19) thus correlating with bax protein expression increase.

![Western blot image]

**Fig. 19:** Down-regulation of bcl-2 protein by 8a and 8b. H1299 and H1299 p53 +/- cells were treated with 100 µM 8a, 8b or left untreated (UC) for 24 hr and bcl-2 protein expression level was determined from whole cell lysates by western blot analysis using mouse monoclonal anti-bcl-2 antibody. β-actin was used as the loading control. Results are representative of 3 independent experiments.
4.3.3 Translocation of mitochondrial cytochrome to the cytosol during 8a and 8b induced apoptosis.

Previous studies have demonstrated that cytochrome c released from mitochondria by bax induces caspase activation resulting in apoptosis. Thus, cytochrome c was analysed by western analysis in both cell lines treated with 8a and 8b to demonstrate if cytochrome c translocated from the mitochondria to the cytosol after treatment. In 8a treated H1299 and H1299 p53 +/- cells, cytochrome c was released into the cytosol from the mitochondria (Fig. 20A) while in H1299 cells treated with 8b there was up-regulation in mitochondrial cytochrome c with a lesser release of cytochrome c into the cytosol. However, in H1299 p53 +/- cells treated with 8b there was up-regulation of cytochrome c release into the cytosol (Fig. 20B).
Fig. 20: Translocation of mitochondrial cytochrome to the cytosol during 8a and 8b induced apoptosis. H1299 and H1299 p53 +/- cells were treated with (+) or without (-) 100 µM 8a (A) or 8b (B) for 24 hr then mitochondrial and cytosolic fractions were separated as described in Materials and Methods. Cytochrome c expression in each fraction was determined by western blot analysis using mouse monoclonal anti-cytochrome c antibody. β-actin was used as the loading control. Results are representative of 3 independent experiments. ‘C Cytochrome c’ represents cytosolic cytochrome c and ‘M Cytochrome c’ represents mitochondrial cytochrome c.

4.3.4 Novel Quinuclidinone derivatives 8a and 8b induce active caspase 9 expression in a time course manner.

Caspase 9 becomes active with the release of cytochrome c and active caspase 9 proteolytically cleaves pro-caspase 3 to active caspase 3 triggering apoptosis. Therefore, experiments were carried out to observe whether 8a and 8b cytochrome c release correlated with up-regulated caspase 9 levels. 8a induced formation of active caspase 9 after 1 hr of treatment but a more significant increase was observed after 12 hr in H1299 cells. In H1299 p53 +/- cells treated with 8a there was a significant change as indicated
by the pixel densities at 1hr but levels remained the same after 6hr as compared to the 0 hr (Fig. 21A). In comparison, 8b induced caspase 9 formation after 6 hr of treatment in H1299 cells and as early as 1 hr after treatment in H1299 p53 +/- cells (Fig. 21B).

Fig. 21: Novel Quinuclidinone derivatives 8a and 8b induce active caspase 9 expression in a time course manner. H1299 and H1299 p53 +/- cells were treated with 100 µM 8a (A) or 8b (B) for the indicated time interval and caspase 9 protein expression level was determined from whole cell lysates by western blot analysis using mouse monoclonal anti-caspase 9 p10 antibody that detects the caspase 9 cleaved fragment. GAPDH was used as the loading control. The numbers between the caspase 9 and GAPDH panels indicate fold change in pixel density values of caspase 9 as compared to its untreated control and normalized to its respective GAPDH pixel density value. Results are representative of 2 independent experiments.
4.4 Novel quinuclidinone 8b has better effect on cells expressing p53.

Quinuclidinone derivative 8b was more effective in H1299 p53 +/- cells as compared to H1299 cells null for p53 as shown by the earlier mechanistic experiments (Fig. 1-11). To further understand the liaison between 8b and H1299 p53 +/- cells we studied its effect on tumor suppressor protein p53. As expected, 8b induces more p53 expression and transcriptional activity than 8a as compared to its untreated control in H1299 p53 +/- cells (Figs. 22-24).

4.4.1 Novel Quinuclidinone derivatives 8a and 8b up-regulate tumor suppressor protein p53.

Changes in expression levels of a key protein involved in apoptosis, p53, after 24 hr treatment of H1299 p53 +/- cells with 8a and 8b was determined using western blot analysis. Compared to the untreated control, p53 protein expression levels increased after both 8a and 8b treatment but more in 8b treated cells (Fig. 22).
Fig. 22: Novel Quinuclidinone derivatives 8a and 8b up-regulate tumor suppressor protein p53. H1299 and H1299 p53 +/- cells were treated with 100 µM 8a and 8b or left untreated for 24 hr and p53 expression level was determined from whole cell lysates by western blot analysis using monoclonal Bp53-12 antibody that detects all forms of p53. β-actin was used as the loading control. Results are representative of 3 independent experiments.

4.4.2 Time course changes in endogenous p53 expression and localization after treatment with 8b.

We next determined the stability of p53 protein in the H1299 p53 +/- cells with 8b treatment at different time intervals by analyzing the protein expression level by western blot and its nuclear localization by immunofluorescence. p53 protein expression increases within 1 hr of treatment and remained stable even after 24 hr treatment (Fig. 23A). Previous studies have shown that when p53 is activated it translocates into the nucleus (Vogelstein et al., 2000). Thus, investigations were carried out to observe whether 8b induced p53 accumulation in the nucleus. In fact, immunofluorescence analysis using anti-p53 antibody revealed that 8b treatment resulted in punctate p53
accumulation in the nucleus of H1299 p53 +/+ cells within 6 hr of treatment and could be
detected after 24 hr treatment when maximum apoptosis was observed, while in the
untreated cells p53 was located in the cytoplasm (Fig. 23B). Altogether these results
suggest that p53 protein was induced, stabilized and translocated into the nucleus in 8b
treated H1299 p53 +/+ cells

(A)  

<table>
<thead>
<tr>
<th></th>
<th>8b</th>
<th>0</th>
<th>1</th>
<th>6</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

H1299 p53 +/+
Fig. 23: Time course changes in endogenous p53 expression and localization after treatment with 8b. H1299 p53 +/- cells were treated with 100µM of derivative 8b for indicated time interval. (A) From whole cell lysates p53 expression was determined using anti-p53 antibody (Bp53-12) by western blot analysis and β-actin was used as the loading control. (B) Treated cells were fixed and labeled with anti-p53 antibody (Bp53-12) followed by FITC-labeled secondary antibody against p53. To visualize the nuclei, the cells were stained with DAPI. The pictures were taken using fluorescent Nikon microscope with a digital camera at 400x magnification. The merged images are respective FITC images superimposed on DAPI images. Results are representative of 3 independent experiments.
4.4.3 Transcriptional activity of p53 using bax.

To evaluate the possible role of 8b induced p53 accumulation in the nucleus, we evaluated p53 transcription activity function by studying its downstream target bax. From previous studies it is known that bax has p53 response elements and its transcription depends on p53 activation. We first evaluated 8a and 8b induced bax promoter activity in both H1299 and H1299 p53 +/- cells and indeed only in H1299 p53 +/- cells treated with 8b was there a significant ($p \leq 0.01$) increase in bax promoter activity as compared to its untreated control (Fig. 24A). We next determined the levels of bax transcript in H1299 and H1299 p53 +/- cells after treating with 8a or 8b and whether it correlates with the bax promoter activity. Real time RT PCR analysis was used to study the fold changes in treated cells as compared to the untreated control and results indicate that there was a significant ($p \leq 0.01$) 3 fold increase in the transcript levels of 8b treated H1299 p53 +/- cells (Fig. 24B). Altogether these results suggest that 8b works better in H1299 p53 +/- cells due to presence of p53 that was induced significantly in these cells and protein expression of bax follows the bax promoter activity and transcript level trends wherein maximum induction of bax protein was observed in H1299 p53 +/- cells treated with 8b.
Fig. 24: Transcriptional activity of p53 using Bax. (A) H1299 and H1299 p53 +/- cells were transfected with luciferase-bax (Bax-Luc) reporter plasmid together with control plasmid and then treated with 100 µM of 8a and 8b for 24 hr or left untreated (UC). Bax luciferase activity in the cells was determined using dual luciferase assay as described in Materials and Methods. The histogram represents the mean ratio of Bax-Luc luciferase activity to the control luciferase activity (± SD) of 2 independent experiments done in triplicate. (B) RNA isolated from H1299 and H1299 p53 +/- cells treated with 8a, 8b or left untreated (UC) were subjected to Real-Time RT PCR analysis using bax and β-actin primers. The histogram represents fold change in bax transcript levels (± SD) as compared to the untreated control normalized with respective β-actin transcript level. Results are representative of 3 independent experiments done in triplicate. Significance as compared to untreated control was * p ≤ 0.05 and ** p ≤ 0.01.
4.5 Gene expression profiling

For a small scale and focused analysis of gene expression upon treatment of 8a and 8b in H1299 and H1299 p53 +/− cells, we used SuperArray apoptosis array to study 84 genes involved in apoptosis that were regulated by the treatment as compared to the untreated control. In the current study only those genes that had ≥ 2 fold change and were significantly (p ≤ 0.05) up or down-regulated as compared to the untreated control were included (Table 2-5).

4.5.1 8a induces pro-apoptotic and suppresses anti-apoptotic genes in H1299 cells

After 24 hr treatment of H1299 cells with 8a, expression of 4 genes involved in apoptosis were changed as compared to the untreated control. Pro-apoptotic genes, caspase 4 (involved in Fas mediated apoptosis) and TNFRSF9 (receptor belonging to the tumor necrosis factor superfamily), were up-regulated (Table 2). Previous studies have demonstrated that myeloid cell leukemia sequence 1, mcl-1, gene is over expressed in NSCLC (Song, Coppola, Livingston, Cress, & Haura, 2005), but in H1299 cells treated with 8a mcl-1 gene expression was down-regulated as compared to the untreated control. Also, X-linked inhibitor of apoptosis, XIAP gene, was down-regulated (Table 2).
Table 2

*Fold change of apoptotic gene expression in H1299 cells (null for p53) after 24 hr treatment of quinuclidinone derivative 8a, as determined by SuperArray Analysis*

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change(@)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN ± SEM</td>
</tr>
<tr>
<td><strong>UP-REGULATED</strong></td>
<td></td>
</tr>
<tr>
<td>Caspase Family</td>
<td></td>
</tr>
<tr>
<td>Caspase 4 (Caspase 4 apoptosis-related cysteine peptidase)</td>
<td>+2 (± 0.01)*</td>
</tr>
<tr>
<td><strong>TNF Receptor Family</strong></td>
<td></td>
</tr>
<tr>
<td>TNFRSF9 (Tumor Necrosis Factor Receptor Superfamily, membrane 9)</td>
<td>+3.48 (± 0.07)*</td>
</tr>
<tr>
<td><strong>DOWN-REGULATED</strong></td>
<td></td>
</tr>
<tr>
<td><strong>BCL2 Family</strong></td>
<td></td>
</tr>
<tr>
<td>Mcl-1 (myeloid cell leukaemia sequence 1) BCL2-related</td>
<td>-13.93 (± 0.07)*</td>
</tr>
<tr>
<td><strong>IAP Family</strong></td>
<td></td>
</tr>
<tr>
<td>XIAP (X-linked inhibitor of apoptosis)</td>
<td>-48.50 (± 0.14)*</td>
</tr>
</tbody>
</table>

\(\@\) Fold change is expressed as treated with 100 µM quinuclidinone derivative 8a group compared to untreated control group; + indicates gene up regulation after treatment; - indicates gene down regulation after treatment; * indicates significant gene regulation (p ≤ 0.05); n=2.
4.5.2 8a induces death domain genes in H1299 p53 +/- cells

In H1299 p53 +/- cells 8a induces the over expression of death domain family of genes *FADD* and especially *TRADD* (fold change of >200) which are recruited by death receptors to transmit the apoptosis signal within the cell from the plasma membrane (Table 3). *APAF-1* gene that is essential for mitochondrial apoptosis signaling was also up-regulated with the treatment (Table 3). Along with the up-regulation of pro-apoptosis transducers, 8a in H1299 p53 +/- cells causes the down-regulation of *bcl-2*, an inhibitor of the mitochondrial pathway and *Gadd45a*, an important marker used for the prognosis of NSCLC (Table 3).

4.5.3 8b significantly induces genes involved in TNF death receptor apoptosis pathway in H1299 cells

In H1299 cells, 8b induces over expression of apoptosis promoting genes, primarily those involved in TNF death receptor apoptosis signaling including *TNF, TNFSF8, card 6, TRAF 4* and more significantly *TRAF 2* (Table 4). 8b also induces *HRK* gene expression, which is a known inhibitor of bcl-2 and *LTBR* gene expression, a plasma membrane receptor that triggers apoptosis when activated. Also, over-expression of 2 anti-apoptosis genes, *XIAP*, an inhibitor of apoptosis, and *IGF1R* gene that is involved in promoting tumorigenesis, occurred with the treatment of 8b in H1299 cells (Table 4).
Table 3

Fold change of apoptotic gene expression in H1299 cells p53 +/- (over expressing p53) after 24 hr treatment of quinuclidinone derivative 8a, as determined by SuperArray Analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change&lt;sup&gt;@&lt;/sup&gt;</th>
<th>MEAN ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UP-REGULATED</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CARDD Family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAF-1 (apoptotic peptidase activating factor 1)</td>
<td>+6.73 (± 0.9)*</td>
<td></td>
</tr>
<tr>
<td><strong>Death Domain Family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADD (Fas (TNFRSF6)-associated via death domain)</td>
<td>+6.28 (± 0.28)*</td>
<td></td>
</tr>
<tr>
<td>TRADD (TNFRSF1A-associated via death domain)</td>
<td>+274 (± 0.8)*</td>
<td></td>
</tr>
<tr>
<td><strong>DOWN-REGULATED</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2 Family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl 2 (B-cell CLL/lymphoma 2)</td>
<td>-2.93 (± 0.9)*</td>
<td></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gadd45a (Growth arrest and DNA-damage-inducible, alpha)</td>
<td>-2.3 (± 0.9)*</td>
<td></td>
</tr>
</tbody>
</table>

<sup>@</sup> Fold change is expressed as treated with 100 µM quinuclidinone derivative 8a group compared to untreated control group; + indicates gene up regulation after treatment; - indicates gene down regulation after treatment; * indicates significant gene regulation (p < 0.05); n=2.
Table 4

Fold change of apoptotic gene expression in H1299 cells (null for p53) after 24 hr treatment of quinuclidinone derivative 8b, as determined by SuperArray Analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change&lt;sup&gt;%&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SEM</strong></td>
<td></td>
</tr>
<tr>
<td><strong>UP-REGULATED</strong></td>
<td></td>
</tr>
<tr>
<td>BCL2 Family</td>
<td></td>
</tr>
<tr>
<td>HRK (Harakiri, BCL2 interacting protein (contains only BH3 domain))</td>
<td>+2**</td>
</tr>
<tr>
<td>CARDD Family</td>
<td></td>
</tr>
<tr>
<td>Card 6 (Caspase recruitment domain family, member 6)</td>
<td>+2.81 (± 0.92)*</td>
</tr>
<tr>
<td>IAP Family</td>
<td></td>
</tr>
<tr>
<td>XIAP (X-linked inhibitor of apoptosis)</td>
<td>+2.7 (± 0.21)*</td>
</tr>
<tr>
<td>TNF Ligand Family</td>
<td></td>
</tr>
<tr>
<td>TNF (Tumor necrosis factor (TNF superfamily, member 2))</td>
<td>+2.43 (± 0.92)*</td>
</tr>
<tr>
<td>TNFSF8 (Tumor necrosis factor (ligand) superfamily, member 8)</td>
<td>+3.0*</td>
</tr>
<tr>
<td>TRAF Family</td>
<td></td>
</tr>
<tr>
<td>TRAF2 (TNF receptor-associated factor 2)</td>
<td>+2 (± 0.5)**</td>
</tr>
<tr>
<td>TRAF 4 (TNF receptor-associated factor 4)</td>
<td>+2 (± 0.8)*</td>
</tr>
<tr>
<td>TNF Family</td>
<td></td>
</tr>
<tr>
<td>LTBR (Lymphotxin beta receptor or TNFR superfamily, member 3)</td>
<td>+3.1 (± 0.8)*</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>IGF1R(Insulin-like growth factor 1 receptor)</td>
<td>+2 (± 0.14)*</td>
</tr>
</tbody>
</table>
Fold change is expressed as treated with 100 µM quinuclidinone derivative 8b group compared to untreated control group; + indicates gene up regulation after treatment; - indicates gene down regulation after treatment; * indicates significant gene regulation (p ≤ 0.05); ** indicates significant gene regulation (p ≤ 0.01); n=3.

4.5.4 8b induces changes in expression of the bcl-2 family of genes in H1299 p53 +/-
cells

Bcl-2 family of genes includes both inhibitors and facilitators of apoptosis. In H1299 p53 +/- cells, 8b induces over expression of bax (bcl-2 associated X protein), bag 3 (bcl2 associated anthanogene 3), bcl2l10 (bcl-2 like 10) bclaf1 (bcl2 associated transcription factor 1), bik (bcl-2 interacting killer) and bnip3l (bcl-2/adenovirus E1B 19 kDa interacting protein like) genes all known apoptosis facilitators (Table 5) along with the down regulation of potent inhibitors of apoptosis, bcl2l1 or bcl-X (bcl-2 like 1), bcl2l2 (bcl-2 like 2) and mcl -1 genes (Table 5). 8b also induces death domain genes expression, FADD and TRADD and over-expression of TNF receptor gene, LTBR gene in H1299 p53 +/- cells (Table 5).
Table 5

Fold change of apoptotic gene expression in H1299 cells p53 +/- (over expressing p53) after 24 hr treatment of quinuclidinone derivative 8b, as determined by SuperArray Analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change(^\circ) MEAN ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UP-REGULATED</strong></td>
<td></td>
</tr>
<tr>
<td>BCL2 Family</td>
<td></td>
</tr>
<tr>
<td>Bag3 (BCL2-associated athanogene 3)</td>
<td>+2.1 (± 0.45)*</td>
</tr>
<tr>
<td>Bax (BCL 2-associated X protein)</td>
<td>+2.83 (± 0.55)*</td>
</tr>
<tr>
<td>BclA1 (BCL 2 related protein A1)</td>
<td>+2.96 (± 0.18)*</td>
</tr>
<tr>
<td>Bcl2l10 (BCL2-like 10) apoptosis facilitator</td>
<td>+2.25 (± 0.45)*</td>
</tr>
<tr>
<td>Bclaf1(BCL2-associated transcription factor 1)</td>
<td>+2.05 (± 0.7)*</td>
</tr>
<tr>
<td>Bik (BCL2-interacting killer) apoptosis-inducing</td>
<td>+2.25 (± 0.16)*</td>
</tr>
<tr>
<td>Bnip3l (BCL2/adenovirus E1B 19kDa interacting protein 3 like)</td>
<td>+36.76 (± 0.9)*</td>
</tr>
<tr>
<td>TNF Family</td>
<td></td>
</tr>
<tr>
<td>LTBR (Lymphotoxin beta receptor (TNFR superfamily, member 3))</td>
<td>+3.65 (± 0.99)**</td>
</tr>
<tr>
<td>Death Domain Family</td>
<td></td>
</tr>
<tr>
<td>FADD (Fas (TNFRSF6)-associated via death domain)</td>
<td>+9.2 (± 0.55)*</td>
</tr>
<tr>
<td>TRADD (TNFRSF1A-associated via death domain)</td>
<td>+660 (± 0.75)*</td>
</tr>
<tr>
<td>Genes</td>
<td>Fold Change</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>DOWN-REGULATED</strong></td>
<td></td>
</tr>
<tr>
<td><strong>BCL2 Family</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bcl2ll (BCL2–like 1)</em></td>
<td>-8 (± 0.41)*</td>
</tr>
<tr>
<td><em>Bcl2l2 (BCL2–like 2)</em></td>
<td>-4 (± 0.93)*</td>
</tr>
<tr>
<td><em>Mcl-1 (myeloid cell leukaemia sequence 1) BCL2-related</em></td>
<td>-9.62 (± 0.97)*</td>
</tr>
</tbody>
</table>

* Fold change is expressed as treated with 100 µM quinuclidinone derivative 8b group compared to untreated control group; + indicates gene up regulation after treatment; - indicates gene down regulation after treatment; * indicates significant gene regulation (p < 0.05); ** indicates significant gene regulation (p < 0.01); n=3.
CHAPTER 5: DISCUSSION

Lung cancer has been identified as the most lethal form of cancer and is classified into two main types, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), based on the invading cell morphology. The only line of treatment for advanced NSCLC is chemotherapy, but resistance to treatment occurs frequently and quickly (Schwartz, 2004; Sekido, Fong, & Minna, 1998). Previously, we reported novel quinuclidinone derivatives, especially 8a and 8b, that showed increased cytotoxicity against NSCLC cell line H1299 null for p53 (Malki et al., 2006). Derivatives 8a and 8b were developed from PRIMA-1 (compound 4) that was found to restore mutant p53 function at a very high concentration in select p53 point mutants (Bykov, Issaeva, Selivanova et al., 2002; Malki et al., 2006). But the preliminary work shows that quinuclidinone derivatives 8a and 8b are effective at a much lower concentration than PRIMA-1 (compound 4) and other quinuclidinone derivatives. Importantly, 8a and 8b have a higher effect against lung cancer cells than normal lung cells. In this study, we show that quinuclidinone derivatives 8a and 8b induced apoptosis and significant accumulation in S phase in H1299 cells regardless of their p53 background. Here we demonstrate that apoptosis occurred as seen by the detection of characteristic events such as DNA fragmentation and activation of caspase 3 with the cleavage of PARP-1. Importantly, in keeping with the preliminary analysis, 8a and 8b were less effective in inducing apoptosis in normal lung epithelial cells, NL-20, thus making them potentially attractive anti-cancer drugs.
Many studies show that cell phase arrest, especially S phase arrest, occurs along with chemotherapeutic-induced apoptosis. Presumably, the S phase arrest helps anticancer drugs target more cells for apoptosis (Fulda & Debatin, 2004; Pucci et al., 2000; Smith et al., 2000). In our analysis, quinuclidinone derivatives 8a and 8b promoted increased apoptosis in lung cancer cell lines at 24 hr after treatment which coincided with the S phase cell cycle arrest. 8a, which is more potent at inducing apoptosis in H1299 cells null for p53, showed increased accumulation in S phase in these cells; similarly 8b, which works well in H1299 p53 +/+ cells, showed an increased S phase arrest in these cells. These results suggest that increased S phase arrest does sensitize the cells to quinuclidinone chemotherapeutic apoptosis.

In this study we show for the first time that treatment of H1299 and H1299 p53 +/+ cells with 8a and 8b caused an up-regulation of sphingomyelinase activity. Interestingly, 8a had a greater effect on H1299 cells null for p53 whereas 8b had more significant effect in H1299 cells transfected with p53. But most importantly, both derivatives did not induce SMase in NL-20 cells. The role of sphingomyelinase activation and subsequent release of ceramide was observed in some of the initial studies of doxorubicin, cisplatin and gemcitabine but it was activated in normal and cancer cells at similar levels (Lacour et al., 2004; Modrak et al., 2004; Morita et al., 2000). Intriguingly, our results contrast previous findings in that sphingomyelinase activity was significantly increased in cancer cells compared to normal cells treated with quinuclidinone derivatives 8a and 8b, an effect rarely seen in chemotherapeutics.
We further characterized SMase activity induced by 8a and 8b using potent neutral and acid sphingomyelinase inhibitors. Evidence from inhibitor studies suggests that both neutral and acid sphingomyelinase were induced by 8a and only neutral sphingomyelinase was induced by 8b in H1299 cells. While in H1299 p53 +/- cells, 8a predominantly induced acid sphingomyelinase and 8b induced neutral sphingomyelinase. These results suggest that SMase is an important upstream regulator for apoptosis induced by 8a and 8b in lung cancer cells and when inhibited, it abolishes the derivatives anti-cancer activity establishing their involvement in cellular response to 8a and 8b. The difference between isoforms induction could be related to 8a and 8b triggering different receptors and also due to presence of different p53 status.

Ceramide, a hydrolysis product of sphingomyelin generated by both neutral and acid sphingomyelinase and a known second messenger of apoptosis (Carpinteiro et al., 2008; Hannun & Obeid, 1995; Jarvis et al., 1994; R. N. Kolesnick & Kronke, 1998), was induced in H1299 and H1299 p53 +/- cells with the treatment of 8a and 8b. Ceramides generation correlates with SMase stimulation in both cell lines by 8a and 8b. In H1299 cells, where both NSMase and ASMase are induced, there is a higher induction of ceramide as compared to the untreated control. Similarly in H1299 p53 +/- cells where 8b stimulates more apoptosis, 8b induces an increased ceramide generation as compared to the untreated control. Ceramide is also generated synthetically by the de novo pathway involving ceramide synthase (Carpinteiro et al., 2008; Hannun & Obeid, 1995; R. N. Kolesnick & Kronke, 1998; Spiegel, Foster, & Kolesnick, 1996). There is no role of de novo synthesis of ceramide in the cellular response of 8a and 8b induced apoptosis as
seen by the inhibition of ceramide synthase having no effect on 8a and 8b anti-cancer activity.

JNK phosphorylation occurs as early as 1 hr after quinuclidinone derivatives 8a and 8b treatment in both cell lines but is at its maximum after 24 hr treatment. 8a mainly induces phosphorylation of 54 kDa isoforms while 8b induces phosphorylation of both isoforms. These results are intriguing and contrasting to PRIMA-1 (derivative 4), which inhibits JNK signaling (T. Wang et al., 2007). JNK is recognized as an inducible target of sphingomyelinase generated ceramide and exogenous ceramide mediated apoptosis, especially in lung cancer (Hannun & Obeid, 1995; R. N. Kolesnick & Kronke, 1998; Kurinna et al., 2004). Studies show that NSMase stimulates protein kinases, JNK pathway being one of the major cascades involved (Raines, Kolesnick, & Golde, 1993). In correlation with previous studies, 8b mainly activates NSMAse with the higher phosphorylation of JNK in both cell lines. 8a phosphorylates JNK more in H1299 cells as compared to H1299 p53 +/- cells. It has been implicated that ASMase may not be important in triggering JNK pathway through the TNF receptor in some cell types which could explain the differences in effect by 8a in H1299 p53 +/- cells where ASMase is mainly induced (Adam, Ruff, Strelow, Wiegmann, & Kronke, 1998).

The sphingomyelinase, ceramide and differential JNK phosphorylation results so far suggest a role of the death receptors in mediating the cellular response from outside to within the cells. This notion was clarified by the procaspase 8 processing results from the treatment of derivatives 8a and 8b in both cell lines. The procaspase 8 levels decreased with increase in treatment time which mimics studies showing that extracellular apoptosis
signal by ligand binding to the death receptors is processed within the cell by the formation of DISC. This involves the death domain proteins and procaspase 8 that triggers the auto-catalytic cleavage of procaspase 8 to caspase 8 (Ashkenazi & Dixit, 1998; Kischkel et al., 1995; Martin et al., 1998). This role will be further assessed in future studies involving receptor binding analysis.

JNK also plays an active role in stimulating bax expression, allowing the release of cytochrome c and activating caspase 9, which cleaves procaspase 3 to effector caspase 3. It also regulates anti-apoptotic proteins such as bcl-2 protein expression (Deng et al., 2001; Lei et al., 2002; Maundrell et al., 1997). Even caspase 8 that is activated directly through death receptor activation can either activate effector caspase 3 directly by proteolytically cleaving procaspase 3 or by triggering the mitochondrial apoptosis pathway through the bcl-2 related family members (Budihardjo et al., 1999). Interestingly, for mitochondrial apoptosis pathway to be triggered, bax levels often increase so that it may bind to the outer mitochondrial membrane to allow the release of cytochrome c. Bax function is inhibited by bcl-2, which binds to bax and prevents it from binding to the mitochondrial membrane (Gross et al., 1998; Oltvai et al., 1993; Wolter et al., 1997). Our results show that after the treatment with derivatives 8a and 8b, bax levels increased at the same time as bcl-2 levels decreased, presumably disrupting the balance between pro- and anti- apoptotic proteins, which would further promote the apoptosis signal. Importantly, cytochrome c levels increased in the cytoplasm with treatment indicating it was released from the mitochondria due change in membrane potential. In the cytoplasm, cytochrome c forms apoptosome complex that causes the cleavage of
procaspase 9 to active caspase 9. Caspase 9 is an important molecule in mitochondrial triggered apoptosis causing the activation of caspase 3, marking the final few steps required for apoptosis to occur (Barinaga, 1998; P. Li et al., 1997; Pan, O'Rourke, & Dixit, 1998). Although chemotherapeutics activate the receptor death pathway, the mitochondria mediated death pathway is also essential. The results so far are indicative of not only an initial activation through the death receptors, but also an active involvement of the mitochondria mediated apoptosis by the novel quinuclidinone derivatives 8a and 8b in these lung cancer cells.

From the mitochondrial apoptosis studies, the most interesting findings were that bax protein expression critically increased in 8b treated H1299 p53 +/- cells along with maximum release of cytochrome c from the mitochondria suggesting a role for the tumor suppressor protein p53 that is over-expressed in these cells. To delve further, p53 protein expression level and cellular localization were studied and the results corroborate increased p53 expression within the nucleus after treatment with 8b. Also when studying the function of p53, the results indicate an increase in transcriptional activity of the bax promoter that has the p53 consensus sequence and there was an up-regulation in the bax gene expression as seen with the quantification of bax transcript level. These results substantiate studies that have shown chemotherapeutics to work better in the presence of functional p53 (Lu & El-Deiry, 2009; Weller, 1998). The p53 activity along with the neutral sphingomyelinase activity are both suggested to be involved in potentiating a better anticancer effect of 8b in this cell type.
After understanding that sphingomyelinase is required for both 8a and 8b molecular mechanism, the next goal was to identify differences or similarities in gene expression pattern associated with these novel derivatives. The gene expression profiling for 8a in NSCLC revealed a different pattern with changing p53 status. 8a in H1299 cells up-regulated caspase 4 expression that is involved in Fas mediated apoptosis and TNF receptor super family 9 (TNFRSF9 also known as CD137 or 4-1BB) expression. TNFRSF9 is a tumor suppressor marker identified for NSCLC (Zborovskaia & Tatosian, 2004) and is an inducible target recognized for anti-tumor immune response, therefore being investigated for immunotherapy (Houot et al., 2009; Sica & Chen, 1999). Here, the results obtained suggest that 8a can be used as an adjuvant for immunotherapy. Along with up-regulation of pro-apoptotic genes, 8a also down regulated key anti-apoptotic genes, mcl-1 and XIAP. Mcl-1 belongs to the bcl-2 family and is involved in inhibiting apoptosis by interacting with pro-apoptotic proteins such as bak (Leu, Dumont, Hafey, Murphy, & George, 2004). In contrast to reports where mcl-1 is up-regulated in NSCLC affecting chemosensitivity (Song et al., 2005), 8a down-regulates the gene. XIAP, also a potent inhibitor of apoptosis that binds to caspases 3, 7 and 9 and prevents their activity (Deveraux & Reed, 1999), is critical for chemosensitivity (Cheng et al.). Therefore in NSCLC, inhibitors of XIAP are used in conjunction with known chemotherapy to increase its effectiveness (Dean et al.). Here, 8a significantly decreases XIAP gene expression by almost 50 fold in H1299 cells.

8a regulates a different set of genes in cells over-expressing p53. Here, the results indicate that the death domain genes, TRADD and FADD, were up-regulated, especially
TRADD (> 200 fold). The over expression of these genes induces acid sphingomyelinase activity through the TNF receptor (Schwandner, Wiegmann, Bernardo, Kreder, & Kronke, 1998). We show that 8a induces acid sphingomyelinase activity in H1299 p53 +/- cells which correlates with increased expression of TRADD and FADD. TRADD transcriptional activation causes apoptosis independent of p53 (Yount et al., 2001); therefore, our results suggest 8a may induce initially a p53 independent apoptosis pathway through the activation of acid sphingomyelinase. But since 8a treatment leads to an up-regulation of p53 protein and an increase in its transcriptional target bax, p53 may also play an important role in 8a apoptosis effect. This is also supported by the up-regulation of APAF-1, another transcriptional target of p53, by the treatment of 8a in the H1299 p53 +/- cells. APAF-1 binds to cytochrome c and recruits procaspase 9 forming an apoptosome that is an essential functional unit for mitochondrial apoptosis to occur (P. Li et al., 1997). Up-regulation of APAF-1, increase release of cytochrome c, and presence of caspase 9 all suggests the mitochondrial pathway is involved in 8a induced apoptosis and is p53 dependent in H1299 p53 +/- cells. Interestingly, Gadd45a also a p53 transcription target is down-regulated in these cells with the treatment. However, here the down regulation of Gadd45a could be related to 8a’s lower effect in these cells compared to H1299 cells and may be p53 independent.

Similar to 8a, 8b also regulates different genes in cells with different p53 status. In H1299 cells that are null for p53, 8b induces mostly genes (TNF, TNFSF8, TRAF2, TRAF4, LTBR) involved in TNF receptor apoptosis signaling (Gaur & Aggarwal, 2003; Hsu, Shu, Pan, & Goeddel, 1996; Kuai et al., 2003; VanArsdale et al., 1997).
Interestingly, in these cells 8b triggers neutral sphingomyelinase that is mainly induced due to the activation of TNF receptor therefore laying a premise for this particular gene regulation observed with 8b treatment. Among extracellular death receptor signaling genes, HRK (DP5), a gene identified in regulating intracellular mitochondrial pathway, was up-regulated with 8b treatment. HRK interacts with bcl-2 and bcl-xL through the BH3 domain thereby preventing their inhibitory activity (Imaizumi et al., 1999). Interestingly, two genes known to inhibit apoptosis, XIAP and IGF1R, were also induced by 8b treatment in H1299 cells, (Cheng et al.; Dean et al.; Riedemann & Macaulay, 2006) thus may contribute towards the lowered effect of the derivative in H1299 cells.

In H1299 p53 +/+ cells, 8b induces the up-regulation of bcl-2 family of genes that are pro-apoptotic (bag3, bax, bcla1, bcl2l10, bclaf1, bik and bnip3l) and down-regulates those that are anti-apoptotic (bcl2l1, bcl2l2 and mcl-1) (Adams & Cory, 1998; Brunelle & Letai, 2009). Bax expression pattern in the array analysis was consistent with the other results presented in this study. This opposing regulation changes the balance between pro- and anti-apoptotic proteins leading to increased apoptosis in these cells. Bcl-2 related proteins are mainly involved in mitochondrial apoptosis. Thus, from the p53, bax, cytochrome c and caspase 9 results along with the array analysis results, a very important role is suggested for mitochondrial apoptosis in 8b mediated apoptosis in NSCLC cells over-expressing p53. Interestingly, similar to 8a, 8b also induces high expression of TRADD and FADD in H1299 p53 +/+ cells. These results taken together suggest that a common receptor may be involved in eliciting 8a and 8b activity in H1299 p53 +/+ cells, such as the TNF receptor, being that TRADD expression is significantly higher than
FADD expression. It is also plausible that p53 is involved in the transcriptional activation of these genes, although this has not been shown before therefore would need further assessment to define this role. 8b activated neutral sphingomyelinase in both cell types and also induced up-regulation of TNFRSF3 (or LTBR) suggesting that TNF receptor is possibly the predominant receptor involved in 8b activity in these cells.

Overall, our findings suggest that quinuclidinone derivatives 8a and 8b caused apoptosis in H1299 and H1299 p53 +/- cells along with S phase arrest. Importantly, 8a and 8b induced apoptosis more so in cancer cells than normal cells by activating sphingomyelinase activity. Our data suggests that the sphingomyelinase mediated apoptosis predominantly involves the TNF receptor super family, as seen by the various results that show different SMase isoform activation depending on the cell type and stimulating ligand. However, the difference in upstream activation gives rise to changes in common downstream effectors involved such as ceramide and JNK. These derivatives activate both the extrinsic and the intrinsic apoptosis pathways. Mitochondrial apoptosis related regulators were involved especially in cells over-expressing p53. These derivatives can work independent of p53 activity but the presence of p53 augments the apoptosis effect. Thus, we have potential novel anti-cancer drugs that may work through the TNF receptor-sphingomyelinase pathway independent of p53 to induce cellular death of cancer versus normal cells (Fig. 25), initiating a path for target based drug development.
Fig. 25: Schematic representation of 8a and 8b induced apoptosis model in NSCLC.

8a and 8b induce apoptosis both by up-regulating sphingomyelinase dependent death receptor apoptosis pathway and through the intrinsic mitochondrial pathway involving the bcl-2 related proteins. Both derivatives work independent of p53 in these cells. 8a induces both neutral and acid sphingomyelinase while 8b induces only neutral sphingomyelinase. The involvement of death receptors was seen by the early processing of procaspase 8. Sphingomyelinase activation triggered the production of ceramide and downstream phosphorylation of JNK. The involvement of intrinsic mitochondrial pathway was seen by the up-regulation of bax, cytoplasmic cytochrome c and caspase 9 and by the down regulation of bcl-2 protein. In cells having functional p53, the activity of
p53 is triggered potentiating its role in the apoptosis. Involvement of 8a and 8b in triggering TNF receptor was substantiated by the gene array analysis and induction of neutral sphingomyelinase activity but this relationship needs to be further assessed.
CHAPTER 6: FUTURE WORK

The trend in the 21st century is developing target based therapeutics that have a lower side effect profile to help in bettering the quality of life of patients. In NSCLC, the main line of therapy is chemotherapeutics; therefore, many efforts are been made to develop new and novel compounds that may in the future be used as therapeutics. From the results obtained so far we have two such potential compounds, novel quinuclidinone derivatives 8a and 8b that need further development. These quinuclidinone derivatives 8a and 8b were derived from a parent compound, PRIMA-1, by modifying the side chains on the parent compound to a benzilidine or acetonide. The derivatives activate sphingomyelinase for their mode of action and the data suggests interaction with death receptors, especially TNF receptors. Confirmation will require a receptor binding assay.

To further assess whether these derivatives have analogous effect in different cancer types with similar p53 profile, some preliminary studies were carried out using head and neck cancers having wild type p53 (JHU012), single amino acid mutation that affects the DNA binding function of p53 (JHU013) and p53 null (JHU011) using MTT assay. As seen from Fig. 26, 8b affected head neck cancer cells independent of p53. In contrast to NSCLC where 8a had a higher effect in p53 null cells, it seems 8a has higher effect in cells having single amino acid p53 mutation in head and neck cancer cells (Fig. 26). Similar to NSCLC studies, 8b does induce bax transcriptional activity higher in cells having wild type p53 conformation (Fig. 27). Therefore, based on these findings from these preliminary experiments it would be pertinent to study whether 8a and 8b can restore the mutant p53 conformation by analyzing more cell lines that were originally studied in PRIMA-1 analysis and also determine if sphingomyelinase is involved in their mechanism.
**Figure A**

Different Qunuclidinone derivative treatments (100µM)

- **JHU012**
- **JHU013**
- **JHU011**

**Figure B**

Different concentration of 8b (µM)

% of viable cells

- **JHU012**
Fig. 26: Cell viability analysis for head and neck cancers with different p53 status.

(A) JHU012, JHU013 and JHU011 cells were treated with 100 µM of 8a and 8b and assessed for viable cells using the MTT assay. The histogram represents the percentage of viable cells (± SD) of 2 independent experiments done in triplicate. (B) The dose dependent curve for JHU012 using MTT assay represented as percentage of viable cells at each dosage (± SD). (C) the dose dependent curve for JHU013 using MTT assay represented as percentage of viable cells at each dosage (± SD). (D) the dose dependent curve for JHU011 using MTT assay represented as percentage of viable cells at each dosage (± SD).
Fig. 27: Promoter activity of p53 using Bax in head and neck cancer cells. (A) JHU011 and JHU012 cells were transfected with luciferase-p53 (p53-Luc) reporter plasmid together with control plasmid and then treated with 100 µM of 8a and 8b for 24 hr or left untreated. p53 luciferase activity in the cells was determined using dual luciferase assay similar to the one described in Materials and Methods. The histogram represents the mean ratio of p53-Luc luciferase activity to the control luciferase activity (± SD) of 2 independent experiments done in triplicate. (B) JHU011 and JHU012 cells were transfected with Luciferase-bax (Bax-Luc) reporter plasmid together with control plasmid and then treated with 100 µM of 8a and 8b for 24 hr or left untreated. Bax luciferase activity in the cells was determined using dual luciferase assay as described in Materials and Methods. The histogram represents the mean ratio of Bax-Luc luciferase activity to the control luciferase activity (± SD) of 2 independent experiments done in triplicate.
activity to the control luciferase activity (± SD) of 2 independent experiments done in triplicate.

The relationship between radiation and derivatives 8a and 8b that showed both synergistic and additive effects needs to be assessed further using sphingomyelinase activity analysis as sphingomyelinase is involved in radiation based apoptosis (Haimovitz-Friedman et al., 1994; R. Kolesnick & Fuks, 2003). Because 8a and 8b up-regulate genes involved in TNF receptor apoptosis, it will be interesting to investigate their use in conjunction with immunotherapy.

It is pertinent to study these derivatives that showed potential in an in-vitro system also in an in-vivo system. Preliminary mouse toxicity studies were done in B6D2F1-J mice from Jackson laboratories at dosages 1, 2.5, 5, 10 mg/kg. These studies did not indicate any toxicity in form of weight loss, dietary intake, behavioral changes, body composition or death. Therefore further assessment using higher dosages to determine the lethal dose would be essential to help in profiling these drugs so that they can be applied in a cancer-mouse model to assess its mechanism in an in-vivo system. In addition, the stability of these compounds in-vivo needs to be assessed using LC-MS.

Most importantly, based on promising results from the current study, future derivatives can be developed that work at a much lower concentration and target sphingomyelinase activity in cancer cells more than normal cells to give rise to a wide range of target based therapeutics.
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APPENDIX A: ABBREVIATION LIST

µM: Micromolar

APAF-1: Apoptotic peptidase activating factor -1 gene
APAF-1: Apoptotic peptidase activating factor -1 protein
Apo-2L: also known as TRAIL; TNF-related apoptosis inducing ligand
Apo-3L: also known as TWEAK; TNF-like weak inducer of apoptosis
Arg 175: Arginine amino acid number 175
ASMase: Acid sphingomyelinase
ATCC: American type culture collection
AU: Arbitrary units
Bad/bad: Bcl-2 associated death promoter gene
Bad/bad: Bcl-2 associated death promoter protein
Bag 3/bag 3: Bcl-2 associated anthanogene 3 gene
Bag/bag: Bcl-2 associated athanogene gene
Bag/ bag: Bcl-2 associated athanogene protein
Bak/bak: Bcl-2 homologous antagonist/killer gene
Bak/ bak: Bcl-2 homologous antagonist/killer protein
Bax/bax: Bcl-2 associated X gene
Bax/bax: Bcl-2 associated X protein
Bcl-10/ bcl-10: B-cell leukemia/lymphoma 10 gene
Bcl-10/ bcl-10: B-cell leukemia/lymphoma 10 protein
Bcl-2/ bcl-2: B-cell CLL/lymphoma 2 gene
Bcl-2/ bcl-2: B-cell CLL/lymphoma 2 protein

*Bcl2l10/ bcl2l10*: Bcl-2 like 10 gene

*Bclaf1/ bclaf1*: Bcl2 associated transcription factor 1 gene

*Bcl-X / bcl-X or bcl2l1*: Bcl-2 like 1 protein

Bcl-X/bcl-X or bcl2l1: Bcl-2 like 1 protein

Bcl-xL/ bcl-xL: B cell lymphoma extra large protein

Bcl-xS/ bcl-xS: B cell lymphoma extra small protein

*BGS*: Bovine growth serum

*BH3*: Bcl-2 homolgy domain 3

*Bid/ bid*: BH3 interacting domain death agonist protein

*Bik/ bik*: Bcl-2 interacting killer gene

*Bik/ bik*: Bcl-2 interacting killer protein

*Bim/ bim*: Bcl-2 like protein 11

*Blk/ blk*: B lymphocyte tyrosine kinase protein

*Bnip3l/ bnip3l*: Bcl-2 adenovirus E1B 19 kDa interacting protein like gene

*bp*: Base pair

*CAD*: Caspase activated deoxyribonuclease

*Card 6*: Caspase recruitment domain family, member 6

*Caspase*: Cysteine dependent aspartate specific proteases

*CLL*: Chronic lymphocytic leukemia

*CMV*: Cauliflower mosaic virus

*DAPI*: 4',6-diamidino-2-phenylindole
DFF45: DNA fragmentation factor 4
DIABLO: Direct IAP binding protein with low pI
DISC: Death-inducing signaling complex
DNA: Deoxyribonucleic acid
DR3: Death domain containing TNF receptor super family, death receptor 3
DR4: Death domain containing TNF receptor super family, death receptor 4
DR5: Death domain containing TNF receptor super family, death receptor 5
dUTP: Deoxyuridine triphosphate
EDTA: Ethylenediaminetetraacetic acid
EGFR: Epidermal growth factor receptor
FACS: Fluorescence- activated cell sorter
FasL: Fas ligand
FasR: Fas receptor also known as CD95, APO-1
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
FLICE: FADD like interleukin-1-β-converting enzyme
FLIP: Flice like inhibitory protein
Gadd45a: Growth arrest and DNA damage inducible protein 45 alpha protein
Gadd45a: Growth arrest and DNA damage inducible protein 45 alpha gene
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GI50: 50% growth inhibition
H1299 p53 +/-: Non small cell lung carcinoma cell line over-expressing p53
H1299: Non small cell lung carcinoma cell line null for p53 derived from a lymphoid

His 175: Histidine amino acid number 175

hr: Hour/hours

HRK (DR5): Harakiri, BCL2 interacting protein (contains only BH3 domain gene

HSV: Herpes simplex virus

IAPs: Inhibitors of apoptosis

IGF1R: Insulin like growth factor receptor 1 gene

IGF1R: Insulin like growth factor receptor 1 protein

JHU011: Head neck epithelial cells null for p53

JHU012: Head neck epithelial cells having wild type p53 expression

JHU013: Head and neck epithelial cells having single amino acid mutation that disrupts

the DNA binding domain

JNK: cJun N terminal kinase

kDa; kilo Dalton

L: Ligand

LiAlH₄: Lithium aluminium hydride

LTBR: Lymphotoxin beta receptor or TNFR superfamily, member 3 gene

LTBR: Lymphotoxin beta receptor or TNFR superfamily, member 3 protein

Mcl-1: Myeloid cell leukemia sequence 1 gene

Mcl-1: Myeloid cell leukemia sequence 1 protein

Met 278: Methionine amino acid number 278

min: Minute/minutes
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NES: Nuclear export signal sequence

NL-20: Normal lung epithelial cells

NLS: Nuclear localization sequence

Noxa; Phorbol-12-myristate-13-acetate-induced protein 1

NSCLC: Non small cell lung carcinoma

NSMase; Neutral sphingomyelinase

PARP-1: Poly [ADP-ribose] polymerase-1

PI: Propidium iodide

PRIMA-1 (derivative or compound 4): p53 reactivation and induction of massive apoptosis-1

Puma: p53 up-regulated modulator of apoptosis

R: Receptor

Real Time RT PCR: Real time reverse transcriptase polymerase chain reaction

RFU: Relative fluorescence unit

RIP: Receptor interacting protein:

RLU: Relative luminescence unit

RNA: Ribonucleic acid

RT: Room temperature

SAPK: Stress activated protein kinase

SCLC: Small cell lung carcinoma

SD: Standard deviation
SMAC: Second mitochondrial-derived activator of caspase

SMase: Sphingomyelinase

TAD: Transactivation domain

tbid: Truncated bid protein

TET: Tetramerization domain

Thr 183: Threonine amino acid number 183

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TNFRSF9: Tumor necrosis factor receptor superfamily 9 gene

TNFSF8: Tumor necrosis factor (ligand) superfamily, member 8 gene

TNFSFR9 (4-1BB or CD137): Tumor necrosis factor receptor superfamily 9 protein

TRAF2: TNF receptor associated factor 2 gene

TRAF2: TNF receptor associated factor 2 protein

TRAF4: TNF receptor associated factor 4 gene

TRAF4: TNF receptor associated factor 4 protein

TUNEL: Terminal deoxynucleotidyl tranferase dUTP nick end labeling

Tyr: 185: Tyrosine amino acid number 185

UV: Ultraviolet radiation

XIAP: X-linked inhibitor of apoptosis gene

XIAP: X-linked inhibitor of apoptosis protein
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