# Imidazolium derived ionic salts induce inhibition of cancerous cell growth through apoptosis

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**Abstract**

Study of imidazolium-based ionic liquids on the 60 human cancer cell lines representing diverse histologies, has identified four compounds which show potency at nano-molar dose.  The Annexin V, DNA Fragmentation, Cell Cycle Effects and Mitochondrial Membrane Permeabilization tests provide insight into their mechansim of action. Also, experiments with A431 human epidermoid carcinoma cells suggest activation of an apoptotic pathway due to activity of the initiator caspase 8 and effector caspase 3.

## 1. Introduction

Toxins by very nature are biodynamic substances as they affect the functioning of the bio-species (victim's body), which also suggests that they could potentially become important source of medicines. [1] Understanding and managing the toxicity of small molecule toxins is a major challenge in the discovery and development of new drugs.[2,3] Once the mechanism of compound toxicities is understood, it can be used to advantage as seen in case of Thalidomide which was withdrawn in 1961 due to its teratogenic effects,[4] but lately it is attracting growing interests for therapeutic uses for cancer and other diseases.[5,6] The development of new anti-cancer agents that reduce the toxicity associated with existing chemotherapies and those targeted at circumventing tumor resistance mechanism is a major focus of drug discovery efforts.[2,3]  Once a new chemical entity has been identified, optimizing its physiochemical properties and convert it into an active pharmaceutical ingredient becomes an important task.  To overcome the challenges of drug solubility, stability, delivery and pharmacological effects the salt formation is a widely used

technique. An estimated half of all the drug molecules used in the medicinal therapy are administered as salts.[5-7] Also, for many decades pharmaceutical industry has successfully employed the approach of combining salts of two drugs to achieve the combined pharmacological effects, as evident from several examples in the literature.[8-15] Recently, similar effort has been touted as ionic liquids-API.[16]  On the other hand, the utility of ionic liquids has been explored in many fields.  However, very little information is available on their toxicity,[17] therefore, interest in the study of their applications in the biomedical field has been limited.  One of the most attractive features of ionic liquids is the potential to tailor their physiochemical properties by changing cation/anion combination, which is not possible for molecular compounds.  Following the adage 'the poison is in the dose', our interest has been to capitalize on this important feature, and explore potential of ionic liquids for biomedical application as useful drugs by manipulating their toxicity.  Earlier, we showed for the first time that ionic liquids have varying cytotoxicity, inhibit the growth of cancerous cells, and therefore, they are excellent candidates for development into useful drugs.[18, 19] Subsequently, we also found that ionic liquids can stop the replication cycle of the Human Imunodeficiency Virus (HIV) by inhibiting the function of HIV-integrase, which is an important enzyme in the virus replication cycle.[20]  Our  initial study of imidazolium-based ionic liquids against the National Cancer Institutes (NCI)'s 60 human tumor cell lines (http://dtp.nci.nih.gov/branches/btb/ivclsp.html) identified four compounds (figure 1a) which are highly active and cause dose dependent effect on the inhibition of cancer cell growth as shown in Figure 1b.  The increased chain length of substituent from twelve to eighteen carbon has increased inhibitory effect on the growth of cancer cells.  In other words lower dose of NSC 747269 is required then NSC 747267 and yet higher inhibition is seen.  Both compounds are chloride salts with only difference in the chain length. Indicating that higher lipophilicity of imidazole makes it more potent against cancer cells. Also, the chloride salt (NSC 747269) is more effective than the bis(trifluoromethanesulfonimide) salt (NSC 747271).
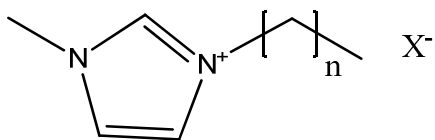
NSC 747260     n =15,  X = Cl
NSC 747267     n =11,  X = Cl
NSC 747269     n =17,  X = Cl
NSC 747271     n =17,  X = $(CF_3SO_2)_2N$

Figure 1a): Structures of Ionic liquids used to study the mechanism of anti-cancer activity
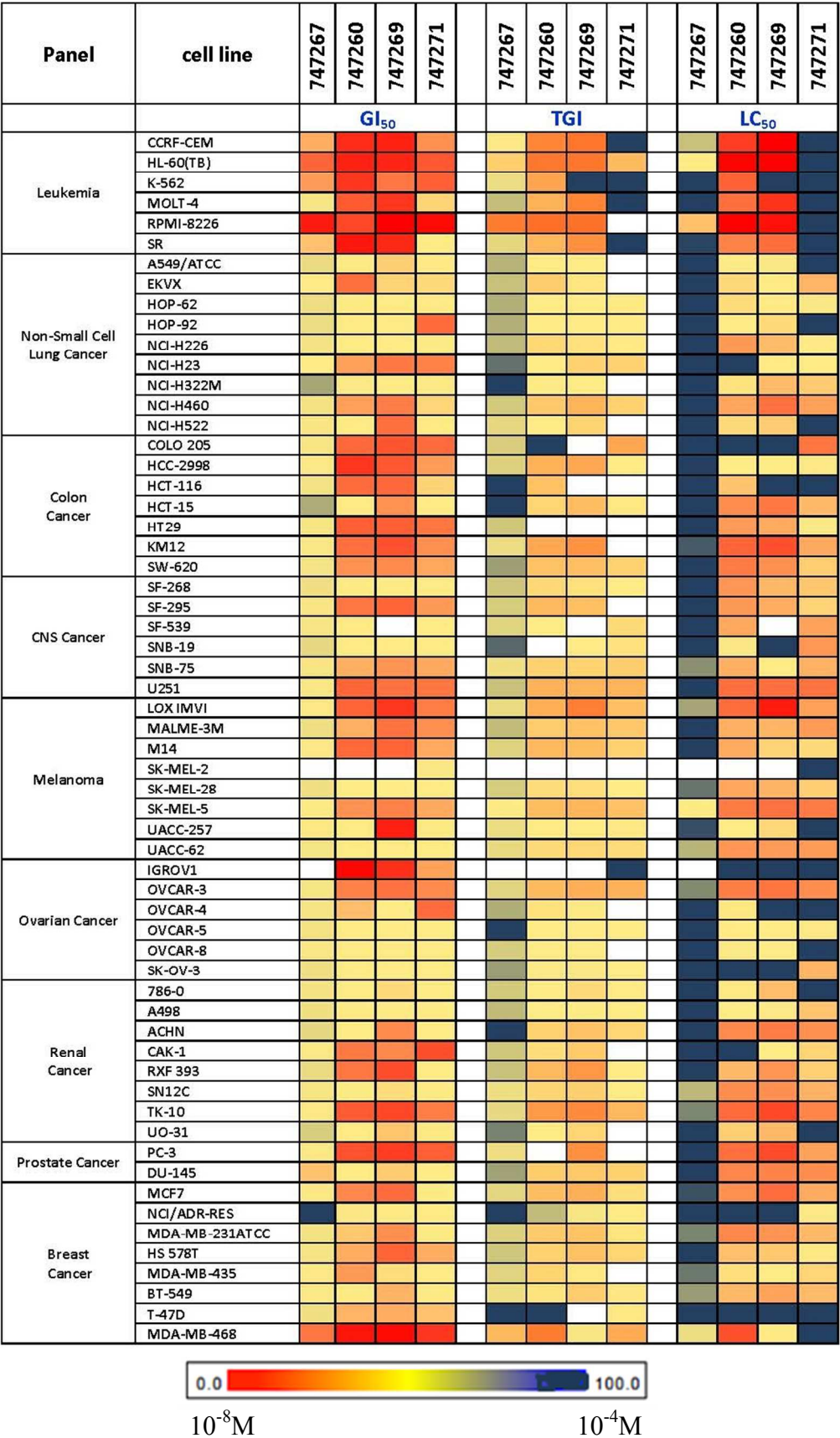
| Panel | cell line | 747267 | 747260 | 747269 | 747271 | | 747267 | 747260 | 747269 | 747271 | | 747267 | 747260 | 747269 | 747271 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | GI$_{50}$ | | | | | TGI | | | | | LC$_{50}$ | | |
| Leukemia | CCRF-CEM | | | | | | | | | | | | | | |
| | HL-60(TB) | | | | | | | | | | | | | | |
| | K-562 | | | | | | | | | | | | | | |
| | MOLT-4 | | | | | | | | | | | | | | |
| | RPMI-8226 | | | | | | | | | | | | | | |
| | SR | | | | | | | | | | | | | | |
| Non-Small Cell Lung Cancer | A549/ATCC | | | | | | | | | | | | | | |
| | EKVX | | | | | | | | | | | | | | |
| | HOP-62 | | | | | | | | | | | | | | |
| | HOP-92 | | | | | | | | | | | | | | |
| | NCI-H226 | | | | | | | | | | | | | | |
| | NCI-H23 | | | | | | | | | | | | | | |
| | NCI-H322M | | | | | | | | | | | | | | |
| | NCI-H460 | | | | | | | | | | | | | | |
| | NCI-H522 | | | | | | | | | | | | | | |
| Colon Cancer | COLO 205 | | | | | | | | | | | | | | |
| | HCC-2998 | | | | | | | | | | | | | | |
| | HCT-116 | | | | | | | | | | | | | | |
| | HCT-15 | | | | | | | | | | | | | | |
| | HT29 | | | | | | | | | | | | | | |
| | KM12 | | | | | | | | | | | | | | |
| | SW-620 | | | | | | | | | | | | | | |
| CNS Cancer | SF-268 | | | | | | | | | | | | | | |
| | SF-295 | | | | | | | | | | | | | | |
| | SF-539 | | | | | | | | | | | | | | |
| | SNB-19 | | | | | | | | | | | | | | |
| | SNB-75 | | | | | | | | | | | | | | |
| | U251 | | | | | | | | | | | | | | |
| Melanoma | LOX IMVI | | | | | | | | | | | | | | |
| | MALME-3M | | | | | | | | | | | | | | |
| | M14 | | | | | | | | | | | | | | |
| | SK-MEL-2 | | | | | | | | | | | | | | |
| | SK-MEL-28 | | | | | | | | | | | | | | |
| | SK-MEL-5 | | | | | | | | | | | | | | |
| | UACC-257 | | | | | | | | | | | | | | |
| | UACC-62 | | | | | | | | | | | | | | |
| Ovarian Cancer | IGROV1 | | | | | | | | | | | | | | |
| | OVCAR-3 | | | | | | | | | | | | | | |
| | OVCAR-4 | | | | | | | | | | | | | | |
| | OVCAR-5 | | | | | | | | | | | | | | |
| | OVCAR-8 | | | | | | | | | | | | | | |
| | SK-OV-3 | | | | | | | | | | | | | | |
| Renal Cancer | 786-0 | | | | | | | | | | | | | | |
| | A498 | | | | | | | | | | | | | | |
| | ACHN | | | | | | | | | | | | | | |
| | CAK-1 | | | | | | | | | | | | | | |
| | RXF 393 | | | | | | | | | | | | | | |
| | SN12C | | | | | | | | | | | | | | |
| | TK-10 | | | | | | | | | | | | | | |
| | UO-31 | | | | | | | | | | | | | | |
| Prostate Cancer | PC-3 | | | | | | | | | | | | | | |
| | DU-145 | | | | | | | | | | | | | | |
| Breast Cancer | MCF7 | | | | | | | | | | | | | | |
| | NCI/ADR-RES | | | | | | | | | | | | | | |
| | MDA-MB-231ATCC | | | | | | | | | | | | | | |
| | HS 578T | | | | | | | | | | | | | | |
| | MDA-MB-435 | | | | | | | | | | | | | | |
| | BT-549 | | | | | | | | | | | | | | |
| | T-47D | | | | | | | | | | | | | | |
| | MDA-MB-468 | | | | | | | | | | | | | | |

0.0 ◼ 100.0

$10^{-8}$M          $10^{-4}$M

Figure 1b)**:** Heatmap pf the antitumor activity $(GI_{50}/\mu M)^a$, TGI and toxicity $(LC_{50}/\mu M)^b$ of imidazolium ILs.

The detail results of drug concentration resulting in Growth inhibition of 50 % $(GI_{50})$ in the measured protein, in total growth inhibition (TGI), and $LC_{50}$ (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) for these compounds are given in the supplementary information. The log mean values of parameter for $GI_{50}$, TGI and $LC_{50}$ related to the log delta values (the maximum sensitivity in excess of the mean) and log range values are given in Table 1 These parameters highlight the selectivity and potency of antitumor agents. Higher values of these deltas and ranges indicate high selectivity against some cancers over others. The lower median log $GI_{50}$ value (-6.36) for **747269** showed it to be the most potent compound for all cell lines. The effective growth inhibition also accounts for its high range log $GI_{50}$ and log $LC_{50}$ values with 1.79 and 2.14 respectively, among all 60 cell lines. Motivated by these results, we have been interested in developing an understanding for their mode-of-action that causes the inhibition of the growth of cancer cells.

**Table 1**. Cytostatic $(GI_{50})^a$ and cytotoxic $(LC_{50})^b$ and $TGI^c$ parameters selected ILs.

| Compd | GI_{50} | | | TGI | | | LC_{50} | | |
|---|---|---|---|---|---|---|---|---|---|
| | Median | Delta | Range | Median | Delta | Range | Median | Delta | Range |
| **747260** | -6.30 | 1.32 | 1.94 | -5.65 | 1.07 | 2.72 | -5.11 | 1.01 | 2.12 |
| **747267** | -5.57 | 1.39 | 2.7 | -4.67 | 1.68 | 2.35 | -4.13 | 1.08 | 1.21 |
| **747269** | -6.36 | 1.21 | 1.79 | -5.74 | 0.89 | 2.63 | -4.99 | 1.15 | 2.14 |
| **747271** | -6.17 | 1.04 | 1.53 | -5.45 | 0.44 | 1.89 | -4.54 | 0.86 | 1.40 |

$^aGI_{50}$: 50% Growth inhibition, concentration of drug resulting in a 50% reduction in net protein increase compared with control cells; $^bLC_{50}$: Lethal concentration, concentration of drug lethal to 50% of cells; $^c$Total Growth Inhibition.

The occurrence of apoptosis, a natural death process in cells is most commonly associated with multiple biochemical events and lead to characteristic cell changes (morphology) and death. These changes include the arrested mitotic activity, decreased DNA replication, fragmentation of DNA, and activation of caspase type enzymes. In order to perform analysis of apoptosis and explore a possible mechanism of action, we studied the effect of all four ionic liquids on the

A431 human epidermoid carcinoma cells. We performed cell cycle changes, DNA fragmentation, mutations, mitochondrial membrane permeabilization and caspase activation. These experiments help to determine activation of key hallmark events which can be indicators of an apoptotic cell death after exposure to the test compounds. The assays are based on the Nucleo Counter NC3000 Instrument (Chemometec, Allerød, Denmark). This system allows for analysis of fluorescence based (multiple fluorophores) assays. The details are described here.

## 2. Materials and Methods

All the ionic liquids were purchased from Merck KgaA (EMD Chemicals), Darmstadt, Germany with purity >95% and were used without any further purification

### 2.1. Compounds Stock Solutions

Compounds were prepared at a 3mM stock concentration by diluting with culture grade DMSO under sterile conditions.

### 2.2. Cell culture

The cell line used in this study was the human epidermoid carcinoma cells A-431 (ATCC CRL-1555). The cells were maintained in RPMI 1640 (ATCC, Manassas VA) containing 10% fetal clone serum (ATCC) supplemented with 1% antimycotic antibiotic solution and 1% kanamycin (Sigma). Cultures were maintained at 37 ºC in a humidified atmosphere of 95% air/ 5% $CO_2$. Cells were seeded (15,000/well) on 96 well plates (NUNC, Rochester, NY) and then exposed to the compounds at ten different doses for 48 h. After the incubation period the cell viability reagent Prestoblue (Invitrogen, Carlsbad, California) was added to each well. Fluorescence corresponding to viable cells was measured using the Fluorostar Optima fluorescence reader (BMG lab tech, Cary, NC) using the standard 485/520nm filters. Results were analyzed using the MARS software (BMG). The determined $IC_{50}$ dose was used on all subsequent experiments.

### 2.3. Annexin V

The annexin V assay has been used as an imaging tool for the detection of phosphatidylserine on the surface of cells, a key event in apoptotic cells.[21] Approximately $3x10^6$ cells were treated with the $IC_{50}$ dose of each compound and controls (DMSO and camptothecin). After exposure, cells

were seeded in triplicates (15,000/well) on 96 well plates (NUNC, Rochester, N Y) stained with annexin V conjugate, and propidium iodide (Biotium, Hayward, CA). Samples where then analyzed using the Nucleo Counter NC3000 (Chemometec, Allerød, Denmark). A one way ANOVA was performed. If significant results were found in the ANOVA, a Post Hoc Test Tukey was also performed.

## 2.4. DNA Fragmentation

DNA Fragmentation as an apoptosis marker is a commonly used assay in drug - cell interaction studies, [22, 23] This nuclease mediated event can be quantified using DNA content and measuring cells containing less than 1DNA equivalent known as Sub - $G_1$. The Nucleo Counter NC3000 system was used for this experiment. The NC3000 assay is based on removal of small DNA fragments and retention of 4',6-diamidino-2-phenylindole (DAPI) stained higher weight fragments. After treatment with ionic liquids at their respective $IC_{50}$'s and implementing the previously described conditions, cells were fixed with ethanol 70%, stained with 1μg/ml DAPI and analyzed by differential image analysis using the NC3000 instrument measuring DAPI intensity. A one way ANOVA was performed. If significant results were found in the ANOVA, a Post Hoc Test Tukey was also performed.

## 2.5. Cell Cycle Effects

Cell cycle alterations can give important insights to a drug's action and can be a useful target against cancer cells.[24] The NC3000 Fixed Cell Cycle assay was performed. This analysis is based on staining cells with DAPI to indicate DNA content. After exposure to test compounds, cells were stained with 1μg/ml DAPI according to the manufacturer's specifications. After staining cells were analyzed with the NC3000 to measure DAPI intensity. A one way ANOVA was performed. If significant results were found in the one way ANOVA, a Post Hoc Test Tukey was also performed.

## 2.6. Mitochondrial Membrane Permeabilization (MMP)

Mitochondria are central in the cell death processes. One of the key events in apoptotic cell death is the permeabilization of the inner mitochondrial membrane.[24,25] The Nucleocounter NC3000 mitochondrial potential was used. After exposure to IC50 dose the $1x10^6$ cells were

stained with 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1**)** and 1µg/ml DAPI according to manufacturer specification and analyzed in the NC3000. A one way ANOVA was performed. If significant results were found in the one way ANOVA, a Post Hoc Test Tukey was also performed.

### 2.7. Caspase 3 y 7

Activation of effector caspases is a key event in the progression of apoptotic cell death.[26] The activation was measured using the Fluorescent Labeled Inhibitors of Caspases (FLICA) probes that bind covalently with active caspase effector enzymes. After treatment as described above, cells were harvested and stained using the green FAM FLICA kit (Immunochemistry Technologies, Bloomington Min.). Samples were then analyzed using the Nucleocounter NC3000 instrument. A one way ANOVA was performed. If significant results were found in the one way ANOVA, a Post Hoc Test Tukey was also performed.

## 3. Results and discussion

To understand the mode-of-action that causes the inhibition of the growth of cancer cells by these compounds we carried out a series of experiments that are described below. Also, as a reference for comparison we used Camptothecin (CPT) a known cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I (topo I)..

### 3.1. Annexin V

Annexin-V is a phospholipid binding protein that belongs to the Annexin family.[27] In the presence of calcium ions it exhibits a high affinity for binding selectively to phosphatidylserine (PS). The PS exposure starts soon after the cell has decided to execute apoptosis. The cell does so well before other features of apoptosis, like nuclear condensation and DNA cleavage, become visible. Cell surface exposure of PS also proceeds while the integrity of the plasma membrane remains uncompromised. These findings strongly suggest that specific machineries are being activated to translocate PS from the inner to the outer leaflet of the plasma membrane during the early phase of apoptosis. Once exposed at the surface PS remains there during the rest of the apoptotic pathway. Therefore, the different phenotypes of apoptosis are characterized by the cell surface exposure of PS. After a 48 h exposure to the $IC_{50}$ dose, the cells presented varied

apoptotic events. These values reflect total apoptotic cells i.e. include both early and late apoptotic cells (Figure 2a). Compounds NSC 747260 and NSC 747271 presented the lesser percentage of apoptotic cells with 12.3% and 15% respectively. Compounds NSC 747267 and NSC 747269 gave high percentage of apoptotic cells with 94.5 % and 89.2% respectively (figure 2b) indicating that cells exposed to these two compounds are undergo significantly high apoptosis (P<0.05).
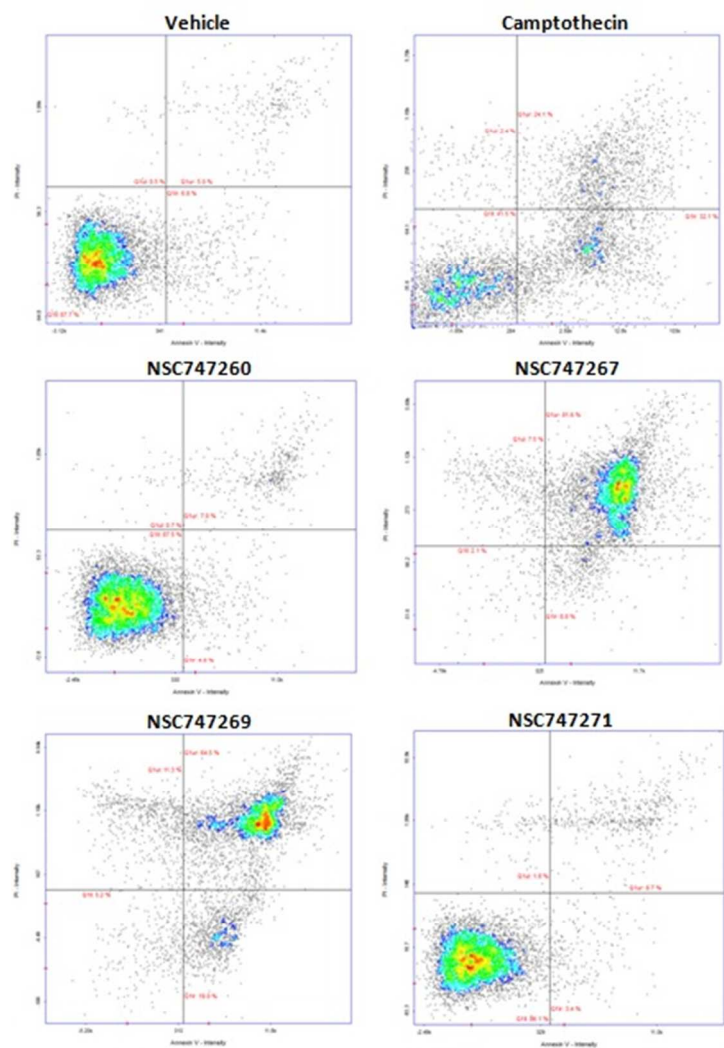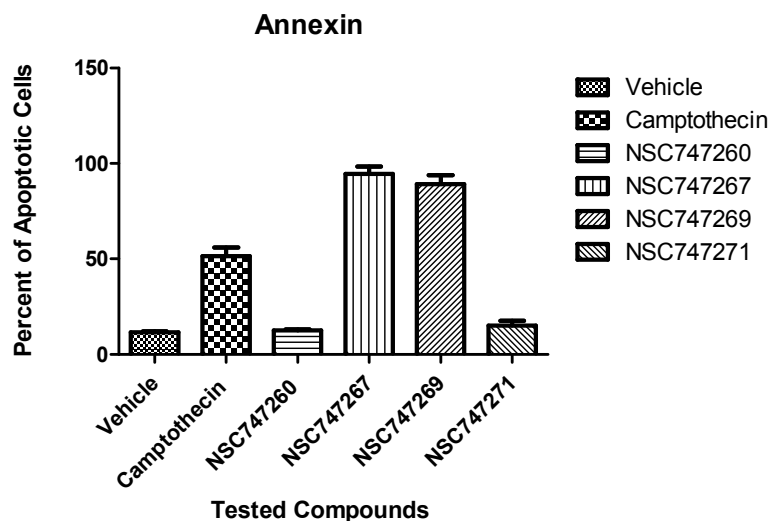


**Figure 2a. Annexin V Results**

**Figure 2b. Annexin Average Percentage of Apoptotic Cells**

## 3.2. DNA Fragmentation

The number of cells with fragmented DNA was measured after 48 h exposure. Figure 3 illustrates the percentage of cells with fragmented DNA. Of all tested compounds only NSC 747269 presented cells with fragmented DNA (Figure 3) with a 30.9% which contrast to NSC 747260 (9.7%), NSC 747267 (13.4%) and NSC 747271 (9.5%). These results demonstrate a sizable difference (P<0.05) of NSC 747269 as compared to other test compounds.
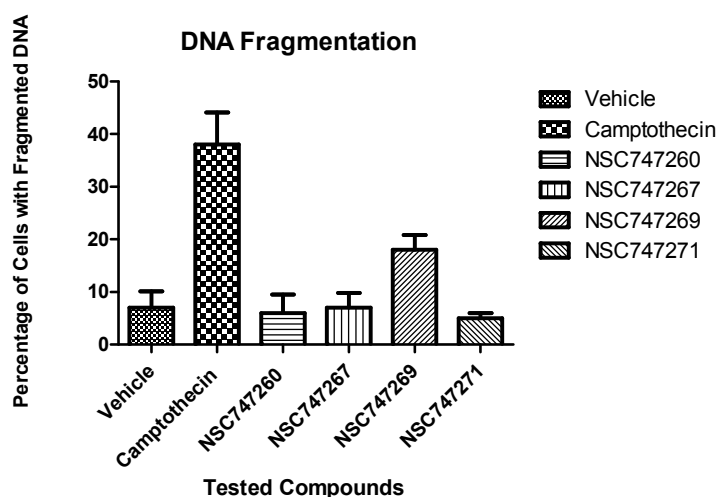


**Figure 3. DNA Fragmentation after Exposure to IC50 Dose**.

*3.3. Cell Cycle Effects*

Assessment of cell cycle effects allows for determination of damage to the cellular replication system after exposure to drugs. Figure 4 presents the percentage of cells arrested at $SubG_0$. These results demonstrated that NSC 747269 exposed cells present an 18% of cells arrested at $SubG_0$ in a significant manner ($P<0.05$) when compared to the negative control. Compounds NSC 747260, NSC 747267 and NSC 747271did not cause cell cycle arrest at $SubG_0$ in a significant manner.
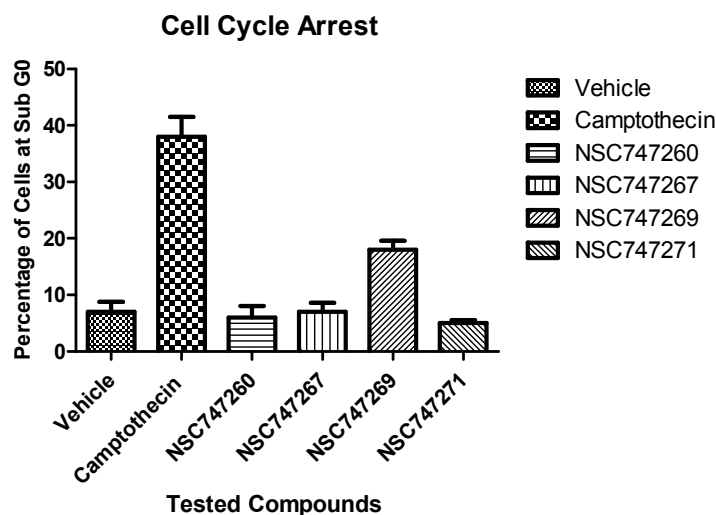


**Figure 4. Cell Cycle Effects**

*3.4. Mitochondrial Membrane Permeabilization*

It is well known that cell death is closely dependent on the structure and functional state of mitochondria,[28] therefore, measurement of alterations in membrane potential can explain important details in drug action mechanism. After 48 h of drug exposure the highest value of 30% was seen with NSC 747260, while effect of other compounds were statistically not significant ($P>0.05$).
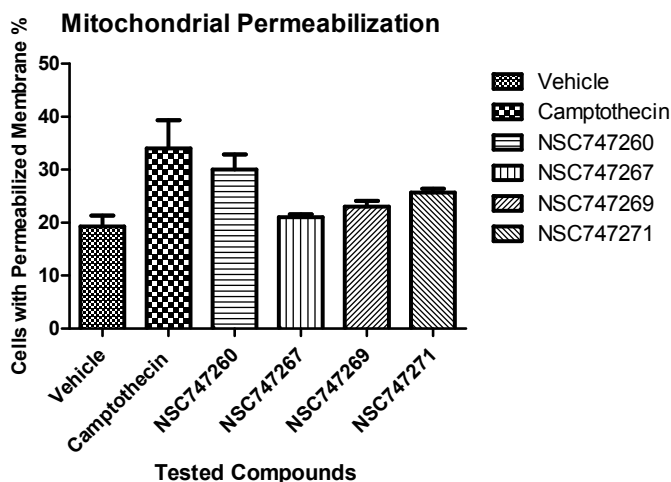
**Figure 5. Mitochondrial Membrane Permeabilization**

### 3.5. *Caspase 3 y 7 activation*

Activation of effector caspases is a hallmark of apoptotic cell death. This event is characterized by changes in membrane morphology, integrity and DNA cleavage, among others.[28] Results obtained after 48 hour exposure were varied, two compounds NSC 747267 and NSC 747269 presented the highest average percentage of apoptotic cells with 84.3% and 76.7% respectively (Figure 6). These values are comparable ($P<0.05$) to the positive camptothecin control. Compounds NSC 747260 and NSC 747271 resulted in the low values of 18.3% and 19.3% respectively. These results suggested that compounds NSC 747267 and NSC 747269 induce activation of effector caspases, and therefore, cells are undergoing an apoptotic cell death.
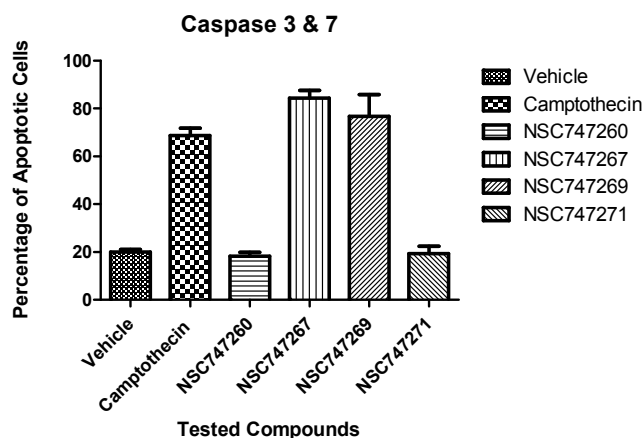
**Figure 6. Caspase 3 and 7 activation**

*3.6.  Caspase 8 activation*

The initiator caspase 8 can be an important precursor in the apoptotic cascade.[29] In our study, after 48 h exposure the cells presented varied activation of caspase 8. Compounds NSC 747260 and NSC 747271 demonstrated the lowest values with 18% and 16% respectively. These values are similar to those obtained in activation of caspase 3 and 7. Compounds NSC 747267 and NSC 747269 showed high activation of caspases with 87.5% and 86.5% respectively. The data suggest that extrinsic apoptotic pathway should be activated by these ionic liquids.
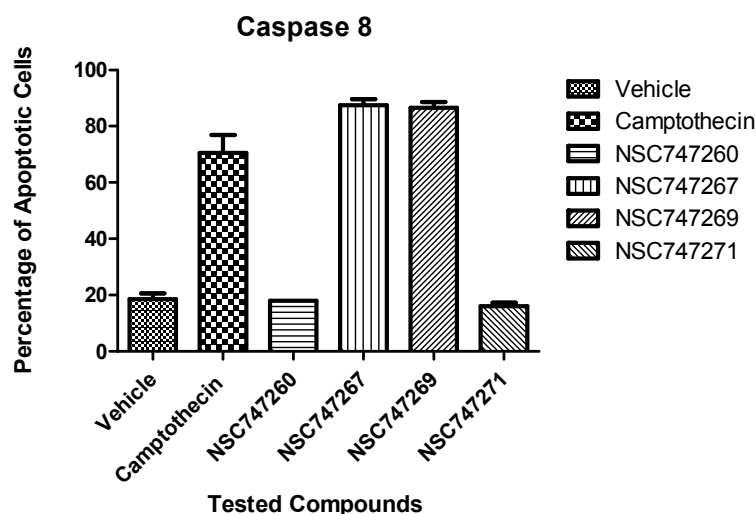


**Figure 7. Caspase 8 activation**

The general tendency of the studied compounds reveals that NSC 747267 and NSC 747269 exposed cells undergo an apoptotic mechanism. These compounds probably activate an extrinsic apoptotic pathway due to activity of the initiator caspase 8.This caspase could cause activation of effector caspase 3.[30,31]  This is further reinforced by the annexin V results which revealed apoptotic cells in compounds NSC 747267 and NSC 747269 but not in NSC 747260 or NSC 747271. Effects on DNA and cell cycle were not significant except on NSC 747269 which revealed some DNA fragmentation and cell cycle arrest. NSC 747260 and NSC 747271 were the most toxic compounds. However, neither revealed significant results among the measured parameters suggesting another cell death mechanism independent from apoptosis.

## 4. Conclusion

In summary, results of this study suggest that two ionic liquids i.e. NSC 747267 and NSC 747269 cause apoptosis in the A431 exposed cancer cells, while NSC 747260 and NSC 747271 although highly toxic, induce a non-apoptotic cell death mechanism. The mitochondrial permeability experiments indicate some activity but lack of statistical significance would render them as inconclusive. Overall this study has deepen our understanding of the potential of ionic liquids against cancer cell growth.

## Acknowledgments

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