Tp53 induces cytotoxicity of cisplatin combined with tunicamycin synergistically in colorectal cancer cells

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Abstract

Effective management of colorectal cancers is often limited by their relative decreasing of response to chemotherapy, partly due to development of resistance against the chemotherapeutic agents or severe side effects of the drugs. To overcome these barriers, multiple drug therapy is a common approach in cancer treatment. We selected two drugs cisplatin and tunicamycin, with unique mode of cytotoxicity, to establish effective dose combinations optimizing the synergism and thus reducing the drug induced side effects. Eight different solutions of cisplatin ranging from 0 µg/ml to 16 µg/ml were evaluated in combination with eight different solutions of tunicamycin ranging from 0 ng/ml to 800 ng/ml by in vitro chemosensitivity assay against two colorectal cancer cell lines HCT116WT and HCT116P53N, where the later lacks the tumours suppressing gene, TP53. The result showed significant difference in chemosensitivity between the two cell lines towards the experimental solutions, where HCT116P53WT was found more susceptible. The IC50 for cisplatin and tunicamycin alone against HCT116WT were found 2.5 µg/ml and 50 ng/ml respectively. The dose combinations of cisplatin to tunicamycin that induce synergism against HCT116WT include 0.25 µg/ml: 25 ng/ml; 0.5 µg/ml: 25 ng/ml; 1 µg/ml: 25 ng/ml and 1 µg/ml: 12.5 ng/ml. The dose combinations for the same against HCT116P53N were revealed markedly high as 2 µg/ml: 25 ng/ml; 2 µg/ml: 50 ng/ml; 4 µg/ml: 12.5 ng/ml and 4 µg/ml: 25 ng/ml, indicating P53 induced cytotoxicity in HCT116WT significantly. To conclude, these findings can serve as strong evidence for p53 role & combination chemotherapy as an effective strategy in cancer treatment with reduced risk of side effects and resistance development.

Keywords: colorectal cancer, cisplatin, tunicamycin, synergy

1. Introduction

According to the report from cancer research UK, bowel or colorectal cancers, where the inner lining of this large intestine may form swollen mass, often known as polyps, are the most common in men and women together that account for more than 50%. This perirectal adenopathy may eventually turns into malignancy since benign adenopathies are not typically seen in this area. In the case of cellular stress like damage of DNA TP53, by transforming from inactive state to the active state, triggers the transcription of a CDR inhibitor gene called P21, thereby halting the cell cycle at G1 phase and gives protection against cancer. However, a mutated P53 gene cannot perform its regulatory function over the cell cycle which is simply devastating, leading to loss of inhibitory control of the cell over the cell cycle. Chemotherapy is one of the main methods especially in metastatic conditions that uses chemical substances like alkaloids, alkylating agents, antimetabolites, antitumor antibiotics, enzymes, hormones etc., constituting a standardized chemotherapy regimen with an aim to cure the condition or in certain stages to reduce the symptoms and prolonging the life span as much as it is possible. Because conventional type of chemotherapeutic agents are primarily based on their ability of killing the rapidly growing cells by interfering with the cell cycle at different stages, the same cell cycle processes during the growth of other normal cell will also be affected and hence are closely associated with common side effects like myelo suppression, haemopoiesis, mucositis and alopecia. Moreover, increasing rate of cancerous cells to become resistant to a particular drug by adopting different cellular mechanisms like actively pumping of the drug out of the cell or alteration of the active site in the drug receptor binding region or detoxification of the drug by expressing more bio transforming enzyme like cytochrome p450 or increased DNA repair imposes a great challenge to eradicate the disease. The consequence of drug resistance ultimately results in rapid deterioration of the disease condition during or shortly after completion of the treatment. One effective but simple way to overcome these problems is to look for drug combinations with different mechanisms that will
make the cancer cell susceptible to the therapy. Most antitumor drug combinations established as clinical setup possess only additive effect, although, in a fewer case combinations result in synergistic activity. Therefore researching for ideal drug combination with good synergistic activity is an exciting field in cancer research. Cisplatin is a novel anticancer adjuvant which can easily form adduct with DNA and protein by attaching to their nucleophilic group that ultimately perturb in the cellular mechanisms, leading to apoptosis [8]. In one study of treating esophageal cancer cells cisplatin was combined with PUMA, a p53 up-regulated modulator of apoptosis (which induction occurs as a result of DNA damage in cell and activate non-functional p53 gene which usually become inactive in cancerous cell due to mutation) was shown to potentiante programmed cell death by increasing the chemo sensitivity of the cancerous cell to the drug [7]. Beside DNA there are other sites in the cell which can be a potential target for anticancer drugs. Endoplasmic reticulum is a vital organelle of the cell and prime site of protein translation and modification and can be targeted for inducing apoptosis by creating ER stress artificially in presence of chemical compound like thapsigargin, tunicamycin, and geldanamycin [9]. In severe and long lasting ER stress, when ER stress cannot be resolved by the first prosurvival approach of the unfolded protein response, the combined effort mediated by ATF4 and ATF6 transcription factors stimulate the expression of CHOP (C/EBP homologous protein, also known as GADD153; growth arrest and DNA damage inducible gene 153, which in turn inhibit the expression of anti-apoptotic protein of Bcl-2 family [10] and increases the expression of other proapoptotic protein Bim [10]. Designing a chemotherapy regimen by combining these two drugs could be an attractive selection. When two drugs are administered concurrently one can affect the pharmacokinetic and/or pharmacodynamics properties of the other drug that ultimately is reflected in the efficacy of drug. Studying drug interaction between two drugs in vitro and at preclinical stage that exert similar effects carries a significant importance, especially in cancer therapy where low therapeutic index and side effects of antitumor drugs are common attributes [11]. This kind of interaction can be demonstrated by any of the three possible consequences. (a) synergism- when the combined effects of two drugs are greater than the sum of the effects of the individual drugs; (b) anergism- when the combined effects of two drugs are lower than the sum of the effects of the individual drugs and (c) additive Effect - when the combined effect of two drugs is equal to the sum of the effect of each drugs given alone. The whole scenario of drug interaction can be graphically presented as introduced by Loewe [12], where the doses of each drugs plotted in a scattered system commonly form a straight “line of additivity” that discerns the additive function from synergistic and antagonistic interactions, known as isobologram. Accordingly, we designed this study to interpret the behavior of cytotoxicity of cisplatin and tunicamycin in combination on two colorectal cancerous cell lines HCT116WT and HCT116P53N, where the later differ from the former by TP53, an apoptosis inducing gene.

2. Materials and methods

2.1 Chemicals, drugs and cell lines, media

PBS, trypsin, TCA, SRB dye, cisplatin & tunicamycin, and media, RPMI-1640, inactivated foetal calf serum (HIFCS), L-glutamine, penicillin G and streptomycin, amphotericin all were obtained from Sigma Aldrich UK. Cell lines HCT116WT and HCT116P53N were collected from cell bank of the School of Life Science, University of Bedfordshire, UK.

2.2 Cell culture of HCT116 WT and HCT116P53N

The HCT116WT and HCT116P53N human colorectal cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (HIFCS), L-glutamine, penicillin G and streptomycin, amphotericin in T75 flasks at 37°C in 5% CO2 and 85% relative humidity.

2.3 In vitro cytotoxicity assay against HCT116P53N cells (using 8000 cells per well)

2.3.1 Plating of cells: For this purpose a working stock cell suspension with cell concentration of 40000 cells/ml, appropriate for each well quantity, i.e., 8000 cells in 200 µl, was made from a primary stock, derived from a preconfluent stock culture of HCT116P53N cells. Then in a 96 well plate 64 wells (8×8) were seeded with 8000 cells in each well by aliquoting 200 µl of working stock and incubated for 24 hours at 37°C, 5% CO2.

2.3.2 Treatment with cisplatin & tunicamycin: Overnight grown HCT116WT cells were treated with eight different concentrations of cisplatin (0 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml& 16 µg/ml) and tunicamycin (0 ng/ml, 12.5 ng/ml, 25 ng/ml, 50 ng/ml, 100ng/ml, 200 ng/ml, 400 ng/ml & 800 ng/ml). An initial stock solution for each drug with a concentration two times higher than the strongest one in the working range (i.e., 32 µg/ml of cisplatin and 1600 ng/ml of tunicamycin) was made for the convenience of work. The layout of drug combinations was designed in such a fashion that well of left bottom corner contains no drug at all; wells in each row contains cisplatin of same strength but increases from bottom to up, whereas wells in each column contains tunicamycin of same strength and increases from left to right. After removing the media from each well appropriate amount of drug solutions were transferred to the respective well to maintain the defined concentration in a final volume of 200 µl. Volume in each well was adjusted to 200 µl by adding media as necessary. The plate was then incubated for 72 hours at 37°C, 5% CO2. The whole procedures were repeated to make three replicates.

2.3.3 Survivor’s estimation: To quantify the alive cells after drug treatment, cells were treated with 50 µl of 50% TCA to fix the cells on well surface followed by staining with 50 µl of 0.4% SRB, washing the unbound dye with 0.1% acetic acid and then resolubilising the cell bound dye by using 150 µl of 10mM Tris. Then 100 µl of aliquot from each well was transferred to a fresh 96 well plate maintaining the unique position of aliquot in new plate. To another four wells 100 µl of Tris was added to counterbalance the effect of Tris and plastic. The intensity of dye in the form of absorbance was measured by scanning with a plate reader, Multiskan Go 1.00.40, at 570nm. The data was then statistically analysed to interpret the effect of drug on cell growth.

2.4 In vitro cytotoxicity assay against HCT116 WT cells (using 8000 cells per well)

The whole procedures involved in this assay, from plating 8000 cells per well to drug treatment and survivor estimation, were identical to that of "In vitro cytotoxicity assay against
HT116P53N cells (using 8000 cells per well)”, except the cell type, which was HT116 WT. The procedure was repeated to make three replicate. All data were recorded for statistical analysis.

2.5. Statistical analysis
The experimental data were presented as the mean ± SEM and were analyzed using the two-tailed Student t test. P < 0.05 was considered statistically significant.

3. Results
3.1 In vitro cytotoxicity assay against HT116P53N cells (using 8000 cells per well)
The cytotoxicity assays of cisplatin and tunicamycin in combination against HT116P53N were performed in triplicate. The average % absorbance of SRB proportional to the survivors after treatment from the three replicates against the drug concentration has been summarised in figure 1 and also depicts the reproducibility of the experiment across the three replicates is represented by standard error of mean. Cisplatin alone within the concentration range 4-8 µg/ml caused cell survival between 40 and 60% and accordingly, the IC50, dose of cisplatin that reduce the cell survival by 50% identified to lie close to 5.5 µg/ml. For tunicamycin, a dose of 100 ng/ml caused an average percent survival of 46.28. So, IC50 should be around 90 ng/ml. In addition, multiple columns heights, belonging to a defined cisplatin and tunicamycin combination that fell between zones that cause cell inhibition between 40-60%. Plot of those corresponding dose combinations that resulted in 40-60% inhibition form the isobologram, as shown in figure 2. By connecting the two points that indicate the IC50 doses for cisplatin and tunicamycin alone, form the linear isobologram that makes a separating zone between synergistic (below the line) and antagonistic (above the line) behaviours of drug combinations. Here, dose combinations like, 2 µg/ml: 25 ng/ml; 2 µg/ml: 50 ng/ml; 4 µg/ml: 12.5 ng/ml reflect synergism, whereas, combinations like 25 µg/ml: 50 ng/ml; 0.5 µg/ml: 50 ng/ml, 1 µg/ml: 50 ng/ml and 2 µg/ml: 12.5 ng/ml reflect antagonism.

3.2 In vitro cytotoxicity assay against HT116 WT cells (using 8000 cells per well)
The cytotoxicity assays of cisplatin and tunicamycin combinations against HT116WT were performed in duplicate. The effects of combinations in each replicate on the viability of HT116WT has been summarised in figure 3, in the form of average % absorbance of the survivors after treatment against drug solutions. The variations between the duplicate assays have been depicted by standard error of mean. According to figure 3, both cisplatin and tunicamycin on their own and in combination within the concentration range (cisplatin: 0-16 µg/ml and tunicamycin: 0-800 ng/ml) used in the assay showed a significant decrease in viability HT116WT when the concentration of drugs were increased with compare to control (i.e., cells not treated with either tunicamycin or cisplatin), which survival was assumed to be 100%. From their single treatment data the IC50 values for cisplatin and tunicamycin were predicted to be around 2.5 µg/ml and 50 ng/ml respectively. A scatter plot of these two IC50 values along with other dose combinations that caused average % survival between 40-60% generates the isobologram (Figure 4). The linear isobologram that passes through the two points of IC50 clarifies the dose combinations that manifest either synergistic (below the line) or antagonistic (above the line) effect (Figure 4). By connecting the two points of IC50 values for cisplatin and tunicamycin alone, form the linear isobologram that makes a separating zone between synergistic (below the line) and antagonistic (above the line) behaviours of drug combinations. Here, dose combinations like, 0.25 µg/ml: 25 ng/ml; 0.5 µg/ml: 25 ng/ml; 1 µg/ml: 25 ng/ml and 1 µg/ml: 12.5 ng/ml reflect synergism, whereas, combinations like 25 µg/ml: 50 ng/ml; 0.5 µg/ml: 50 ng/ml, 1 µg/ml: 50 ng/ml and 2 µg/ml: 12.5 ng/ml reflect antagonism.

3.3 Comparison of cytotoxic effects of cisplatin & tunicamycin combination between HCT116WT & HCT116P53N
The comparative cytotoxic effect of cisplatin and tunicamycin in combination between HT116WT and HCT116P53N cells has been studied and can be graphically presented as in Figure 5. It shows that in all experimental drug combinations, except very few deviations, HCT116WT cells were found more susceptible than HCT116P53N, because, the average % survivals of HCT116WT are clearly lower than HCT116P53N at a particular dose combination.

4. Discussions
Effective management of colorectal cancers, as like of many other cancers, is often limited by their relative decreasing of response to chemotherapy, due to acquired resistance and/or severe side effects. Multiple drug therapy is a common approach in improving individual drug response. Accordingly, cisplatin and tunicamycin were evaluated in eight different predefined dose combinations against two colorectal cancer cell lines namely HCT116WT and HCT116P53N, where the later differ from the first one by lacking a tumours suppressing gene, TP53.

4.1 In vitro cytotoxicity assay against HCT116P53N cells (using 8000 cells per well)
The cytotoxicity assays of cisplatin and tunicamycin combinations against HCT116P53N were performed in triplicate. The average % absorbance of three replicates against drug concentrations as plotted in figure 1, demonstrate that all dose combinations were effective in inhibiting the cancer cell in different proportion. Each treatment has been labelled with associated standard error of mean, as shown in figure 1. Although, in a few cases there are some significant variation in the percent survival of HCT116P53N within a particular dose combinations, overall the result showed reproducibility of the experiment across the three replicates. Variations in some cases can be explained by the same facts as discussed below. We used cell line with two different passage numbers for three replicates. First two replicates were made from one primary HCT116P53N stock (passage no. Pn), whereas, third replicate was from a different primary stock, (passage no. Pm). Each time when cells replicate from parent cell there is chances of mutation, which may results in genetically modified offspring. In cancer cells where mutation rate is high, may render a mutant cell resistant to the drug, which otherwise was sensitive before. If we look at the figure from individual replicate test this may become apparent.

In general, the first two replicates roughly show a linear

~280~
decrease in % survival with increase of concentration (figure 6.i & 6.ii), whereas, in the 3rd replicate (figure 6.iii), at comparatively low dose combinations (cisplatin from 0-0.25 µg/ml and tunicamycin, 0-50 ng/ml) the percent survival is almost close to 100%, however, beyond these concentrations the percent survival declined with increase of concentration. So, it seems that cells in the third replicate became a bit resistant to lower dose combinations and became sensitive again as the dose increases.

In some studies, the IC50 of cisplatin and tunicamycin both alone and in combination with other cytotoxic drugs have been reported. According to Rantanen et al the results obtained from in vitro chemosensitivity testing vary considerably and because of different methods, different cell lines and exposure times they cannot be directly compared [13]. However, the results obtained in this experiment are comparable to results done in other studies. The IC50 against HCT116P53N for cisplatin was found around 5.5 µg/ml when used alone and 2 µg/ml and between 0.5 & 0.25 µg/ml, when used in combination with 25 ng/ml and 100 ng/ml tunicamycin respectively. In one study Hiss et al reported IC50 of cisplatin against UWOV2 ovarian cancer cell line as 4.07 µg/ml alone, but, 0.24 µg/ml in combination with tunicamycin [14]. Both these IC50 values are close to our experimental IC50 and also presenting a clear evidence of -

4.2 In vitro cytotoxicity assay against HCT116 WT cells (using 8000 cells per well)

The cytotoxicity assays of cisplatin and tunicamycin combinations against HCT116 WT were performed in duplicate and except some gross variations the results show reproducibility across two repeat tests. Like the previous experiment, the experimental dose combinations inhibited cell growth in a linear fashion with increase of concentration (figure 3). Clearly, a linear relationship between reductions of average % survival with increase of drug concentration is found, when we consider the individual drug treatment, although the combination treatments demonstrate a mixed behaviour. Cisplatin alone with its highest concentration, 16µg/ml, achieved a significant decrease in HCT116WT viability with a percentage of survival (21.52%), which was 17.48% in case of tunicamycin. However, in combination form, with their highest concentration did not result in highest inhibition of cells (avg. survival percentage of 16.80%), but it was found with dose combination of 4 µg/ml cisplatin and 100 ng/ml tunicamycin with average survival percentage of 15.32%. The IC50 against HCT116WT for cisplatin was found around 2.5 µg/ml when used alone, which is much lower than that against HCT116P53N (5.5 µg/ml) and 1 µg/ml and between 0.5 & 0.25 µg/ml, when used in combination with 12.5 ng/ml and 25 ng/ml tunicamycin respectively. Zhang et al compared cisplatin versus carboplatin against colorectal cancer and reported cisplatin IC50 as 11 ± 2.0 µmol/L which is equivalent to 3.3 µg/ml [15]. This is much closer to the IC50 value obtained in this experiment against HCT116WT. Pairing with tunicamycin also reduced the IC50 from 2.5 to a lowest of between 0.5-0.25 µg/ml which is a clear sign of cisplatin induction. Similarly the IC50 of tunicamycin alone was found at about 50 ng/ml, which reduced to 12.5 ng/ml when combined with 1 µg/ml cisplatin and 25 ng/ml with 0.5-0.25 µg/ml cisplatin, a clear indication of synergism.

4.3 Comparison of cisplatin and tunicamycin combination between HCT116WT and HCT116P53N

The comparative diagrams of cytotoxic effect of cisplatin and tunicamycin combination between HCT116WT and HCT116P53N in the form of average % absorbance vs. different dose combinations of cisplatin & tunicamycin, as drawn in Figure 5 that showed a clear distinctive cytotoxic effects by the drug combinations on HCT116WT over HCT116P53N. Except some overlapping at a few points the graph belonging to the HCT116WT cells (blue colour) reside beneath the curve for HCT116P53N (red color), suggesting that the % survival rate of wild type cell at a particular dose pair is lower than the P53 mutant. In other words, both cisplatin and tunicamycin alone and in combination were more active against HCT116WT than HCT116P53N. Some key features of the diagram are tabulated under table 1. Here the average % survival of both cell line in three different mode (highest, 50% & lowest) with the corresponding dose of a particular drug both in single and combined form are shown. For example, 0.25 µg/ml cisplatin was least effective against wild type that resulted in highest average survival, 78.91%, however, the same cisplatin concentration resulted in far more average survival in P53 mutant cell. Similarly, the IC50 for cisplatin against wild type and P53 mutant are seen as 2.5 µg/ml and 5.5 µg/ml. This is also true with tunicamycin and in their combined form. So, from this comparative analysis it is clear that HCT116WT was more sensitive than HCT116P53N to the experimental dose combinations of cisplatin and tunicamycin.

This differences in sensitivity to antitumor drug between the cells is mainly due for TP53 gene, because HCT116WT cell contains the TP53 gene whereas HCT116P53N lacks the gene. TP53 is well known for its tumor suppressing activity. TP53 expresses p53 protein which plays a vital role in cellular signal transduction pathway that induces apoptotic cycle in the abnormal cell. So this is obvious that cells with P53 will be more susceptible to cytotoxic action of the drug and cells that lack P53 will be less susceptible to cytotoxic action of the drug. This experiment also certifies this fact. Pivonkova et al. confirmed in their studies that P53 protein binds with cisplatin-DNA adduct with great affinity and induce apoptotic cycle rapidly [16], whereas it shows less affinity to the cisplatin-DNA complex in P53 mutant cell.
Fig 1: The chart, plotted as average % absorbance vs. different dose combination of cisplatin & tunicamycin, against HCT116P53N, categorized in eight different tunicamycin concentration group (0, 12.5, 25, 50, 100, 200, 400 and 800 ng/ml) each combined with cisplatin within the concentration range 0-16 µg/ml and represent their associated standard error of mean.

Fig 2: The isobologram formed by plotting the dose of cisplatin in x-axis against the corresponding dose of tunicamycin in y-axis that reduced the HCT116P53N survivors between 40-60% with compared to control (the untreated cells).

Fig 3: The chart, plotted as average % absorbance vs. different dose combination of cisplatin & tunicamycin, against HCT116P53WT, categorized in eight different tunicamycin concentration group (0, 12.5, 25, 50, 100, 200, 400 and 800 ng/ml) each combined with cisplatin within the concentration range 0-16 µg/ml and represent their associated standard error of mean SEM.
Fig 4: The isobologram formed by plotting the dose of cisplatin in x-axis against the corresponding dose of tunicamycin in y-axis that reduced the HCT116WT survivors between 40-60% with compared to control (the untreated cells).
Fig 5: The diagram, plotted as average % absorbance vs. different dose combinations of cisplatin & tunicamycin, categorized in eight different tunicamycin concentration group (0, 12.5, 25, 50, 100, 200, 400 and 800 ng/ml) with all cisplatin doses, compares the cytotoxic effects for particular dose sets between HCT116WT & HCT116P53N cells. Keys: —— Stands for HCT116WT & —— Stands for HCT116P53N cells.

Fig 6 [(i), (ii), (iii)]: The cytotoxic effect of cisplatin and tunicamycin in combination in the form of percent survival upon drug treatment, represented by % absorbance versus drug concentration in triplicate. (i) replicate 1; (ii) replicate 2 and (iii) replicate 3.
### Table 1: Comparison of cisplatin and tunicamycin induced cytotoxicity between HCT116WT and HCT116P53N

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<th><strong>HCT116WT</strong></th>
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<td>Single form</td>
<td>Combined form</td>
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<td>Cisplatin (µg/ml)</td>
<td>Tunicamycin (ng/ml)</td>
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<td>Highest avg. % survival</td>
<td>0.25 (78.91%)</td>
<td>12.5 (68.15%)</td>
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<td>50 % of survival (IC50)</td>
<td>2.5 (50%)</td>
<td>50 (50%)</td>
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<td>Lowest avg. % survival</td>
<td>16 (21.52%)</td>
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### 5. Conclusion
In conclusion, we found that combination chemotherapy using cisplatin and tunicamycin against wild type colorectal cancer cell lines was more effective which induced cytotoxicity synergistically at particular dose combinations. To make this knowledge more informative it requires a detailed study. For example, in P53 mutant or null cell cisplatin is less active. So we need new strategy to make the resistant cell susceptible to the drug. We found that P53 can bring a big difference in cancer therapy. So to make a resistant cell susceptible, it can be transformed first with a pre-programmed vector construct bearing TP53 and subsequent exposure to the drug like cisplatin to which the cell is resistant. Finally, comparing with a control we can decide whether the strategy is effective or not. We can also apply molecular technique to know the extent of expressed P53 and correlate it with cisplatin and tunicamycin activity. In addition, tunicamycin as an ER stress inducer proved to be effective to kill cancer cell. Because, tunicamycin is not still used as an anticancer drug, understanding its mechanism at molecular level could be helpful in designing new analogue with enhanced activity.

### 5. Acknowledgments
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### 6. References