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ARTICLE TYPE

Fluorinated thiazolidinols cause cell death in A549 lung cancer cells *via* PI3K/AKT/mTOR and the MAPK/ERK signalling pathways†

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

A series of 2-imino-4-(trifluoromethyl)thiazolidin-4-ol derivatives were synthesized from one pot, three component reactions of primary amine, aryl isothiocyanate and 3-bromo-trifluoromethyl acetone *via in situ* generation of both symmetrical and unsymmetrical thioureas. All the synthesized derivatives were screened for their *in vitro* anti-cancer activity against human cancer cell lines. Compounds **8l**, **8r**, **8s**, **8t** and **8u** exhibited potent anticancer activity in lung cancer cells (A549) than the other three cell lines. Data obtained from analysis of cell cycle showed that treatment of lung cancer cells with these compounds resulted in G0/G1cell cycle arrest. Studies to understand the molecular mechanism of action of these compounds suggest that the compounds inhibit PI3K, pAkt and mTOR protein expression with concomitant up regulation of tumor suppressor PTEN. These compounds contributed to LC-3 mediated cytoplasmic vacuolation leading to cell death in lung cancer cells. Overall, these compounds modulate the cell death processes by acting *via* inhibition of PI3K/Akt/mTOR pathway and MEK/ERK pathway, key growth factor signalling pathways implicated in abnormal cell proliferation. These molecules can therefore be further tested in *in vivo* models as a potential regimen for efficacy and effectivity in lung cancer.

Introduction

Lung cancer is the most common cause of cancer related mortality. Despite significant research progress there remains many obstacles in development of effective therapy against lung cancer. Cancer chemotherapy is limited by various drug resistance mechanisms. The cellular response towards cytotoxic drugs is regulated by cross-talk between oncogenic signalling pathways and resistance mechanisms. Oncogenic signalling pathways are typically activated by over expression of tyrosine kinase receptors or permanant activation of downstream kinase pathways such as the PI3K/ Akt and MEK/ MAPK pathways which result in malignancy *via* inhibition of cell death process[1]. Phosphatidylinositol-3-kinases (PI3Ks) are lipid kinases that regulate diverse cellular processes including proliferation, survival and motility. PI3K signalling is implicated in various diseases such as cancer, chronic inflammation, allergies and metabolic disorders[2]. In addition, aberrant activation of PI3K signalling confers resistance to conventional therapies. Thus, PI3K is an attractive molecular target for novel anti-cancer therapeutics against many cancers including lung cancer[3,4]. In most cancer cells, elevated activity of PI3K/mTOR is observed. In addition, RAS/RAF/ERK pathway is activated which acts in parallel to promote cell survival. Combinations of MEK/ERK inhibitors with inhibitors of PI3K/mTOR have proved to be more effective than single agents in mouse models of *KRAS*-driven lung cancer[5,6].

In mammalian cells, different types of programmed cell death have been identified[7]. Among them type-I and type-II cell deaths are found to be major processes. Type I cell death corresponds to apoptotic cell death characterized by cell shrinkage, chromatin condensation and degradation of nucleosomal DNA of cells into apoptotic bodies. In contrast, type-II cell death or autophagic cell death is characterized by appearance of double or multiple membrane cytoplasmic vesicles engulfing cytoplasm or cytoplasmic organelles such as mitochondria and endoplasmic reticulum. Furthermore, these autophagic vesicles and their contents are destroyed by lysosomes[8]. Other than these two mechanisms there are mechanisms such as non apoptotic, non autophagic cell death characterised by LC-3 mediated extensive cytoplasmic vacuolation.

It is well known that the introduction of fluorine atom or fluorinated functional group at a specific position is of much interest in modern era of medicinal chemistry for modulating the physical and biological properties of the molecule[9].

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† The authors declare no competing interests.

Electronic Supplementary Information (ESI) available: [Spectral data of Compounds **8a–v,** and experimental procedures for synthesis and biological evaluations]. See DOI: 10.1039/b000000x/

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DOI: 10.1039/C5MD00603A

Trifluoromethylation is one of the most significant strategies to improve pharmacological activities of the molecule. This is due to its high lipophilicity, which enhances *in vivo* uptake as well as transport of molecules to the active site of human body. Thus, fluorine chemistry has got lot of demand in drug discovery[10,11].

In view of the fact that heterocyclic compounds such as thiazolidines, thiazolines and thiazolidinones are considered privileged structural fragments in medicinal chemistry which account for their broad pharmacological activities and possess affinity for various biological targets[12-14], the search for new strategies towards the latter entities is of much significance. 2-Imino-1,3-thiazolidines and unsaturated thiazolines are known in pharmaceutical chemistry for their anti-inflammatory, antimicrobial, antihistamic, antihypertensive, anticonvulsant, anodyne, anti-Alzheimer properties[15,16]. In particular, thiazolidinones are peroxisome proliferator activated receptor (PPAR-receptor) agonists with antineoplastic and antitumor properties[17,18]. One such example is 5-bromo-3-[(3-substituted-5-methyl-4-thiazolidinone-2-ylidene hydrazono]-1H-2-indolinones **1** (Fig. 1) which have been investigated for their primary cytotoxic activity. It was observed that thiosemicarbazone derivatives of indolinones show promising cytotoxicity[19]. Among the 4-thiazolidinone compounds, **2** and **3** (Fig. 1) possess high antimitotic effect *in vitro* in submicromolar concentrations (7 and 10 μ M) and are characterized by low *in vivo* toxicity[20,21]. Micacocidin **4**, a natural product based thiazolidine is a highly cytotoxic compound with metal chelating capability, isolated from the cultured broth of *Pseudomonas sp*. which also showed the anticancer activity[22,23].

In continuation of our studies on development of novel antitumor agents[24-26] and keeping in mind the biological importance of thiazolidines, thiazolines and thiazolidinones, we planned to insert a trifluoromethyl group into the thiazolidine ring to achieve better activity. Therefore, in the current study we have synthesized and evaluated the biological activities of certain novel 2-imino-4-(trifluoromethyl) thiazolidin-4-ol derivatives *via* one pot, three component reaction and by a catalyst free method. Biological evaluation indicated that the effective compounds have the ability to induce LC-3 mediated cell death in lung cancer cells and act as inhibitors of PI3K/Akt/mTOR and MEK/ERK pathway as well.

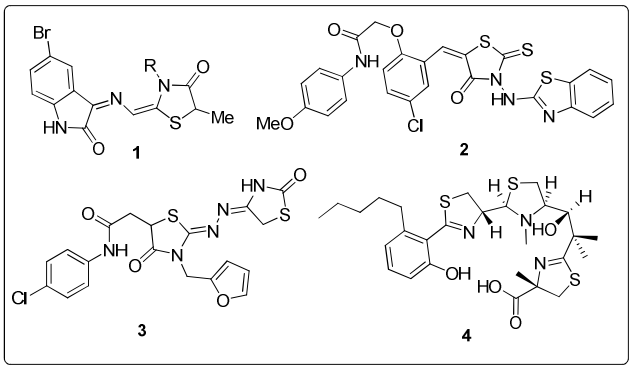
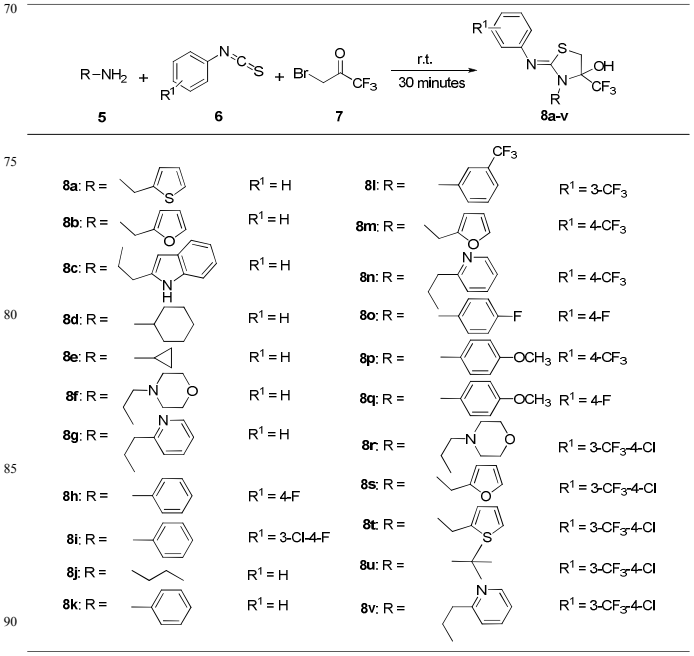


Fig. 1 Biologically active thiazolidine derivatives

Results and discussion

Chemistry

The 2-imino-trifluoromethyl thiazolidinol derivatives (**8a-v**) were synthesized by the reaction of various amine, substituted phenylisothiocyanates and 3-bromo-1,1,1-trifluoromethyl propanone in DMF as solvent at room temperature[27]. The reaction proceeded through *in situ* generation of symmetrical and unsymmetrical thioureas, as an exothermic reaction at room temperature between aromatic and aliphatic amines and substituted phenylisothiocyanates, followed by reaction with 3-bromo-1,1,1-trifluoromethyl acetone, resulting in formation of product **8a-v** (scheme 1).



Scheme 1 Synthesis of 2-imino-trifluoromethyl thiazolidinol derivatives

Biological evaluation

2-imino-trifluoromethyl thiazolidinol derivatives affect cell viability. Cancer cells exhibit uncontrolled cell proliferation while anti-cancer drugs show effect on cell viability and cause death of the cancer cells by various mechanisms. Thus, in order to study the plausible cytotoxic role of these molecules on A549, MDA-MB-231, MCF-7 and HeLa, these cancer cell lines were treated with the compounds for 24h and the cell viability was determined using MTT assay. The PI3K inhibitor LY294002(Ly) was used as the standard. We observed that the compounds **8l**, **8r**, **8s**, **8t**, **8u** have shown strong cytotoxicity than others in the series tested. Interestingly, these active molecules have -CF_3 in R^1 position that might be a contributing factor for the cytotoxic effect. Among all compounds, **8l** has shown significant effect on cell viability of A549 cells (Table 1).

Table 1 *In vitro* cytotoxicity of 2-imino-trifluoromethyl thiazolidinol derivatives (**8a–v**) against A549, MDA-MB-231, HeLa and MCF-7 cancer cells.

	IC_{50} (μM)			
Cmpds	**A549**	**MDA-MB-231**	**HeLa**	**MCF-7**

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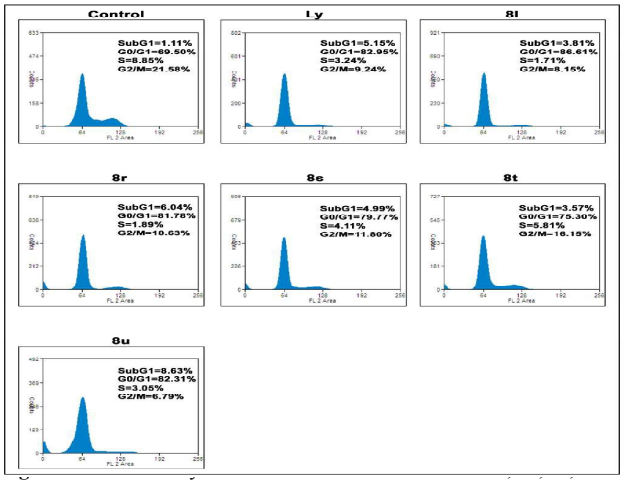
8a	11.05 ±0.37	23.13 ± 0.92	24.78 ±0. 95	33.76 ± 1.88
8b	8.26 ± 0.82	21.57 ± 1.77	24.82 ± 1.53	29.41 ± 1.42
8c	10.61± 0.65	20.58 ± 1.24	27.71 ± 0.82	26.62 ± 0.81
8d	13.77± 0.43	22.48 ± 1.05	28.16 ± 0.97	30.13 ± 1.45
8e	15.08± 0.38	22.70 ± 1.52	24.61 ± 0.88	29.22 ± 0.62
8f	21.22± 1.27	23.82 ± 0.98	26.12 ± 1.61	37.36 ± 2.76
8g	20.05± 0.88	21.51 ± 1.05	28.48 ± 1.72	25.85 ± 1.78
8h	17.12± 0.29	19.55 ± 0.88	32.37 ± 2.54	25.83 ± 1.42
8i	16.83± 0.33	19.02 ± 0.85	19.94 ± 1.09	22.88 ± 0.91
8j	14.32± 0.53	18.66 ± 0.99	24.42 ± 1.55	22.48 ± 0.85
8k	18.61± 0.93	20.94 ± 1.23	29.41 ± 1.43	25.04 ± 1.67
8l	**2.84 ± 0.27**	**3.32 ± 0.45**	**3.57 ± 0.38**	**6.693 ± 0.82**
8m	13.11± 0.84	21.66 ± 0.63	19.68 ± 1.27	25.08 ± 0.91
8n	12.77± 0.09	20.85 ± 0.88	21.0 ± 0.77	25.33 ± 1.56
8o	14.81± 0.55	21.27 ± 0.94	30.74 ± 1.65	34.06 ± 2.78
8p	15.23± 0.76	20.62 ± 1.09	16.83 ± 1.47	29.25 ± 1.34
8q	4.58 ± 0.34	6.64 ± 0.64	6.98 ± 0.34	28.65 ± 0.89
8r	4.15 ± 0.82	5.92 ± 0.22	6.24 ± 0.91	18.24 ± 0.78
8s	3.82 ± 0.55	5.12 ± 0.89	5.84 ± 0.76	15.14 ± 0.56
8t	3.57 ± 0.07	4.48 ± 0.17	6.41 ± 0.62	11.44 ± 0.83
8u	3.26 ± 0.42	4.03 ± 0.34	5.54 ± 0.29	32.34 ± 0.66
8v	6.02 ± 0.77	19.59 ± 1.02	7.12 ± 0.08	11.33 ± 0.27
Ly	**17.7 ± 1.20**	**9.7 ± 0.98**	**20.5 ± 1.45**	**29.1 ± 1.76**

Cell lines were treated with different concentrations of the compounds as described in Materials and Methods. Cell viability was determined by MTT assay. Each of the compound was tested in triplicates (n=3). IC_{50} values are indicated as mean +/- SD of three independent experiments.

Effect of 2-imino-trifluoromethyl thiazolidinol derivatives on Cell Cycle distribution

Cell cycle is regulated by 2 classes of genes- the oncogenes and the tumor suppressors. Dysregulation of these genes results in loss of control over rate of cell cycle progression which ultimately ends up in abnormal cell proliferation. Thus, cell cycle plays a key regulatory role to control abnormal cell proliferation. Cell cycle arrest by anticancer agents is mainly by control of rapid cell proliferation. Thus, we have examined the effect of these derivatives on cell cycle of A549 cells. Flow cytometry results indicated that compounds **8l** (86.61%), **8r** (81.78%), **8s** (79.77%), **8t** (75.30%) and **8u** (82.31%) show G0/G1 cell cycle arrest as indicated by increased percentage of cells in this phase when compared to untreated control cells.



8u at 4 µM or LY 294002 (**Ly**) at 10 µM concentration and incubated for 24h. This was followed by FACS analysis. Compounds exhibited G0/G1 cell cycle arrest as shown by increased percentage of cells in this phase and a decrease in percentage of cells in the G2/M phase. LY 294002 (**Ly**) treated A549 cells have shown G0/G1cell cycle arrest as expected.

LY 294002 (**Ly**) is a standard PI3K inhibitor (Fig. 2) known to cause G0/G1 arrest (82.95%). The FACS data clearly suggests that the compounds block the cell cycle at G0/G1phase in A549 cells.

2-imino-trifluoromethyl thiazolidinol derivatives induce LC-3 mediated cytoplasmic vacuolation leading to cell death in A549 lung cancer cells

Apart from apoptotic cell death, recent studies have indicated vital role of autophagic cell death as well as LC-3 mediated cytoplasmic vacuolation leading to cell death in cancer cell proliferation[7,8]. In order to explore the mechanism by which 2-imino-trifluoromethyl thiazolidinol derivatives induce cell death in A549 lung cancer cells, hoescht staining was performed which indicated that the fluorinated Thiazolidinols had obvious effect on cell cycle arrest but not on DNA fragmentation. Both the morphology (Fig. 3a) and subG1% did not provide evidence of apoptosis. Interestingly, compounds **8l**, **8s** and **8t** have exhibited clear vacuole formation than others. Further, western blot analysis of microtubule associated protein light chain 3(LC-3) which is considered as an important marker of vacuole mediated cell death (Fig. 3a), confirmed LC-3 mediated cell death in cells treated with the compound. LC-3 is expressed as a polypeptide, and is subsequently cleaved to form LC3-I. LC3-I is converted to LC3-II *via* addition of phosphatidylethanolamine (PE) group to the C-terminus[28]. Interestingly, we have observed an increase in the levels of LC3-II in compound treated cells (Fig. 3b) when compared with control untreated cells. Further we also checked the levels of one key autophagy associated protein Atg-7 in control and treated A549 cells which showed an increase in the Atg7 protein levels in treated A549cells compared to control.

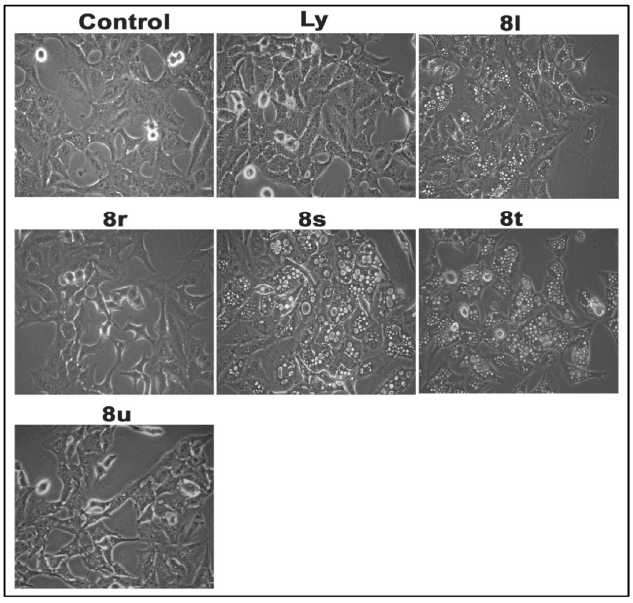


Fig. 3 (a) Effect on cell morphology. Compound treated cells lost their morphology and have shown vacuole formation (20X)

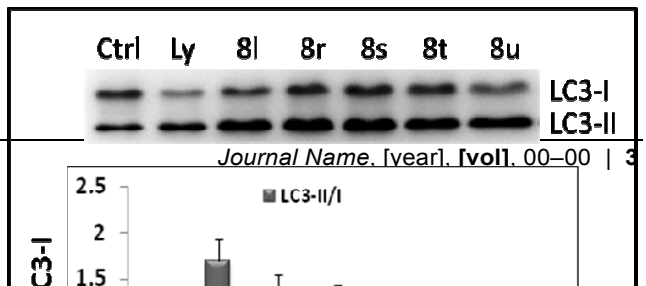


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Fig. 3 (b). Western blotting indicating the levels of vacuole marker LC3 in control and treated cells. During LC-3 mediated cell death, microtubule associated protein LC3 is cleaved and there is an increase in the levels of LC3-II. Here the graph shows the relative density of LC3-II against LC3-I expressed as arbitrary units. X axis represents control and treated samples while Y axis represents LC3-II/ LC3-I ratio.

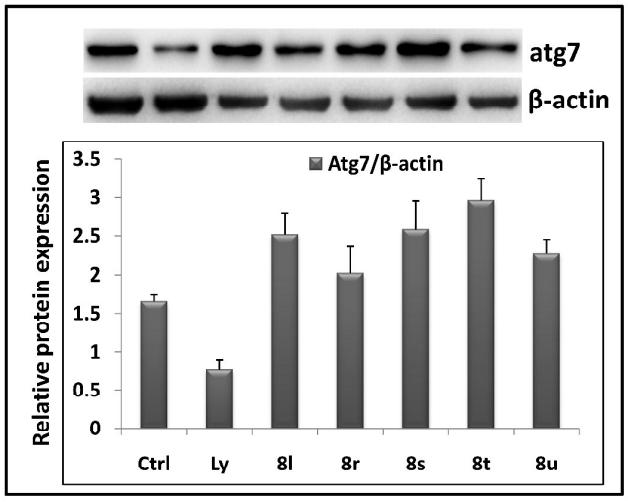

Fig. 3 (c). Western blotting analysis of the key autophagy associated protein Atg7 in control and treated A549 cells. Autophagy is controlled by autophagy-related genes, Atg7 represents a key gene among these. Here the graph shows the relative density of Atg-7 protein against β-actin expressed as arbitrary units. X axis represents control and treated sample while Y axis represents Atg-7/ β-actin ratio.

Effect on PI3K/ Akt/mTOR pathway

Further, we made an attempt to explore the molecular mechanism involved in LC-3 induction by these compounds. The stimulation of class I PI3-Kinase activity inhibits vacuolated cell death whereas class III kinases convert phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and promote the process[29]. Recent studies have indicated possible involvement of PI3K, Akt (PKB) and mTOR in vacuolation mediated cell death[30,31]. Therefore, herein we have investigated plausible effect of these compounds on PI3K and its downstream targets such as Akt and mTOR. We observed down regulation of PI3K, Akt , phosphorylated form of Akt (Akt Ser 473) and mTOR in compound treated A549 cells (Fig. 4a)[32-34].

Since cancer cell proliferation is controlled by various signalling pathways we checked the effect of these analogues on RAS/ MEK/ ERK pathway also. This signalling has strong influence on aggressiveness and rapid proliferation of cancer cells[35]. A549 cells were treated with the compounds and western blot was performed using antibody specific to phosphorylated form of MEK and ERK. We checked the levels of pMEK1/2 and pERK1/2. We observed decrease in the levels of pMEK1/2 and pERK1/2 revealing that these compounds act on both PI3K signalling and MEK/ERK signalling as well. It is well reported that some cues in the cross talks[36] which are effectors of PI3K are also effectors of MEK/ERK signalling (Fig.4b).

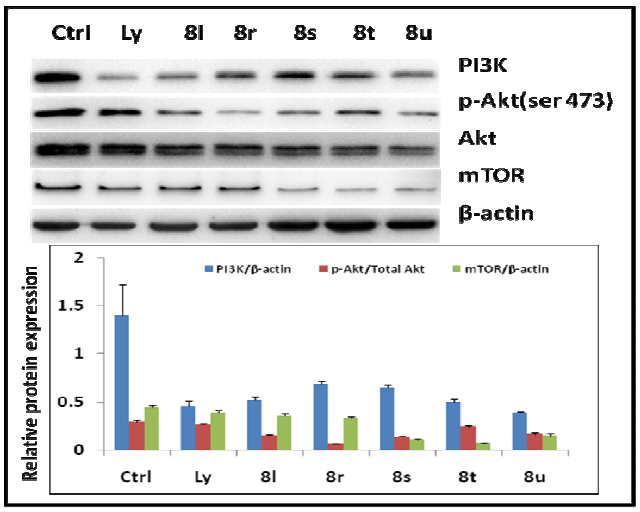

Fig. 4(a) Effect on PI3K/Akt/mTOR pathway.
A549 lung cancer cell line was grown in 60mm dish for 24h. Then cells were incubated with the compounds (Fluorinated Thiazolidinols) at 4μM or with Ly at 10 μM concentration for 24h. The lysates were harvested, quantified by Bradford method and were subjected to western blot analysis. Here, antibodies against PI3K, Akt, pAkt (ser473) and mTOR were used. X axis represents control and treated samples while Y axis represents the relative protein expression expressed as arbitrary units in each case i.e., relative density of PI3K, mTOR against β-actin and pAkt relative to total Akt. β-actin was used as a loading control. Compounds reduced the expression of PI3K, Akt, p-Akt and mTOR significantly.

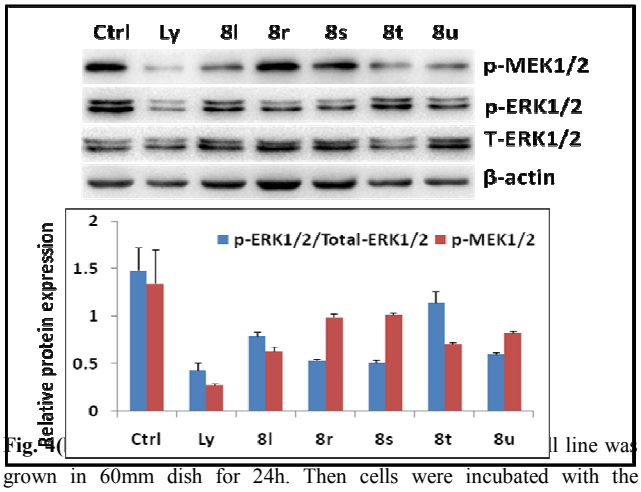

Fig. 4(b) A549 lung cancer cell line was grown in 60mm dish for 24h. Then cells were incubated with the compounds (Fluorinated Thiazolidinols) at 4μM or with Ly at 10 μM concentration for 24h. The lysates were harvested, quantified by Bradford method and were subjected to western blot analysis using the indicated antibodies against pMEK 1/2 and p-ERK1/2. X axis represents control

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and treated samples while Y axis represents the relative protein expression expressed as arbitrary units in each case. Upon compound treatment we observed a decrease in the levels of pMEK1/2 (relative density against β-actin) and pERK 1/2 (relative density against total ERK1/2).

Effect on PTEN

PTEN is considered to be an important candidate tumor suppressor gene that is mutated and lost in many cancers and functions as a buffer for PI3K. Thus, the loss and mutation of PTEN in various cancers leads to hyperactive PI3K signaling. In addition, tumor suppressor protein PTEN (phosphatase and tensin homologue) is known to promote cell death by inhibiting PI3K, Akt (PKB) and finally mTOR[30,31]. Immunocytochemistry was performed in order to understand the plausible effect of compounds on PTEN expression. We noticed increase in PTEN expression (PTEN foci in red by Cy-3 staining captured using confocal microscope) in compound treated A549 cells than control cells (Fig. 5). This result was confirmed by observing the PTEN protein expression in compound treated cells. Merged image indicates increased expression of PTEN foci (red) in cells with nucleus stained in blue colour by DAPI.

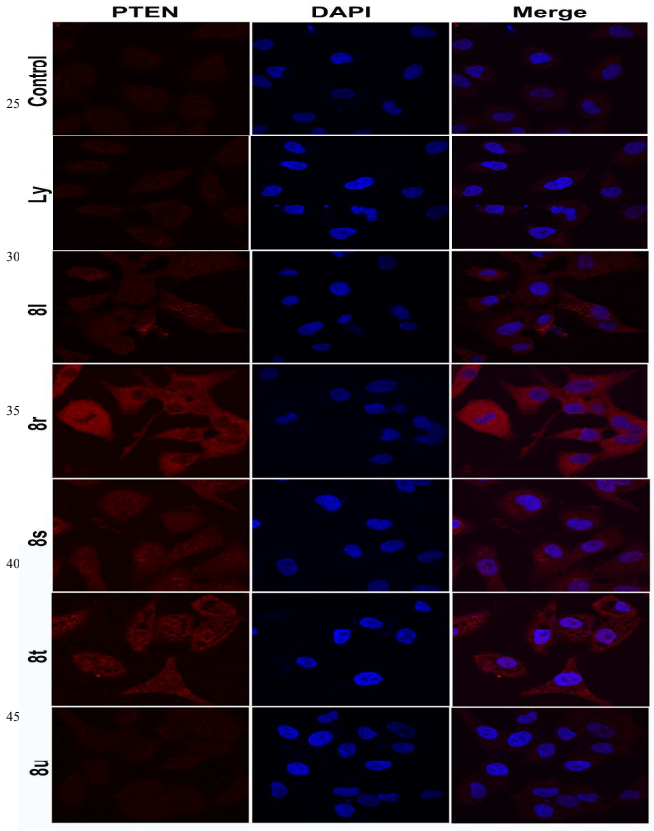

Fig. 5 Effect on PTEN tumor suppressor. A549 cells were treated with the compounds at 4 μM and LY 294002 (**Ly**) at 10 μM concentration and incubated for 24h. The cells were immunostained with PTEN antibody. The PTEN-Cy3 staining was captured using confocal microscope. Red: PTEN, Blue: Nucleus (DAPI), Merge: PTEN+ DAPI.

Discussion

Lung cancer is the most common cause of cancer related death in the world with very limited therapeutic options. Therefore, new therapeutic strategies are urgently needed. Heterogeneity relating to cancer treatment has been observed in multiple malignant cancers, including lung cancer. Apart from classical cytotoxics there is a strong demand to identify small molecules that target growth factor mediated signalling pathways such as PI3K/Akt/ mTOR as well as MEK /ERK signalling.

Cancer cells exhibit uncontrolled cell proliferation while anti-cancer drugs show effect on cell viability and cause death of the cancer cells. In order to examine the cytotoxic activity, the compounds were tested in A549 (human lung cancer cells-p53 wild-type), MDA-MB-231 [(human breast cancer cells (non-functional p53)], HeLa (p53 wild-type) and MCF-7 (p53 wild-type) cells. The MTT assay was carried out in order to determine the cell viability after compound treatment. Out of all the cell lines tested the compounds were effective in the order of A549 >MDA-MB-231> HeLa>MCF-7. Among all the compounds **8l, 8r, 8s, 8t, 8u** were found to be effective (Table 1). Further we investigated the possibility of effect of these compounds on cell cycle as many anti-cancer agents function by inducing cell cycle arrest in cancer cells. Flow cytometry data obtained revealed G0/G1 cell cycle arrest by these compounds (Fig. 2). We also examined the morphological features of A549 lung cancer cells during the treatment with these compounds. We found formation of vacuoles in compound treated cells. These results were further confirmed by determination of expression levels of microtubule associated protein LC3. As expected, we observed an increase in cleaved product of LC3 (i.e. LC3-II). This further confirms the vacuole inducing nature of these compounds (Fig. 3). Also we observed increased protein expression levels of Atg7, a key autophagy associated protein in treated A549 cells compared to control. Earlier in hepatoma cells, by knockdown of the Atg7 expression, it was found that autophagy was inhibited by the reduction of LC3-II expression[37].

Further, we have examined the effect of these compounds on key signalling pathways. Phosphoinositide 3-kinase (PI3k) pathway is an important signalling pathway that integrates metabolism and cell survival/growth signals. The Phosphoinositide 3-kinases (PI3Ks) are a family of proteins involved in various cellular processes such as regulation of growth, proliferation, metabolism, glucose homeostasis and vesicle trafficking. The aberrant function of downstream targets of PI3K such as Phosphatase and Tensin homolog deleted on chromosome (PTEN), Akt, mTOR were found to be associated with the development of malignancies. Interestingly, these compounds have shown decrease in the expression of PI3K, Akt (ser473) and mTOR with concomitant increase in PTEN tumor suppressor in A549 cells. We also observed that these compounds decrease the expression of MEK/ ERK. This indicates that these compounds act as dual inhibitors of PI3K/mTOR[38] and also the MEK/ ERK pathway. Thus, these compounds can be further assessed *in vivo* for tumor regression ability. Also based on these results, these compounds can be considered to have potentiality for further clinical investigations.

Conclusion

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Our study clearly shows that thiazolidinol derivatives suppress lung cancer growth as indicated by cytotoxicity data. Compounds also induced cytoplasmic vacuolation in lung cancer cells as evident by studies on vesicle formation and cleavage of microtubule associated protein LC3 into LC3-I and LC3-II. Interestingly, these compounds have the ability to inhibit proteins of the PI3K/Akt/mTOR pathway and concomitantly activate PTEN. Now, many anti-cancer agents that act on PI3K signalling or mTOR or PTEN are under clinical trials. Further our study suggests that the identified compound targets PI3K/Akt/mTOR and the MEK /ERK, the key signalling pathways and has potential to be used in combinatorial regimen in chemotherapy to overcome the resistance of lung cancer cells to chemotherapeutics.

Acknowledgements

This work was supported by grants from Council of Scientific and Industrial research (XII five year plan projects- DITSF (CSC-0204) and SMiLE (CSC0111)), Department of Science & Technology (SR/ FT/CS-031/2010) and Department of Biotechnology (BT/Bio-CARe/08/511/2010-11, DBT), India. T.D. thanks UGC, A.N., Y.R. and S. H. thank CSIR, New Delhi for the award of fellowship.

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DOI: 10.1039/C5MD00603A

Fluorinated thiazolidinols cause cell death in A549 lung cancer cells *via* PI3K/AKT/mTOR and the MAPK /ERK signalling pathways

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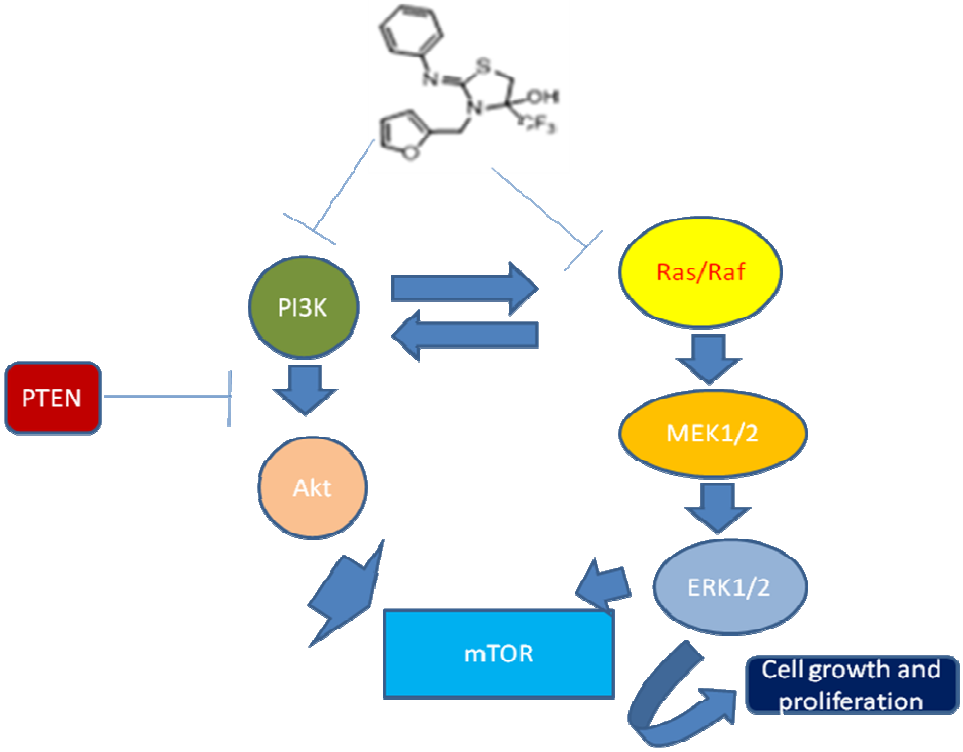

Graphical Abstract

Fluorinated Thiazolidinols act via inhibition of PI3K/Akt/mTOR, and the MEK/ERK signalling pathways and lead to LC-3 mediated cytoplasmic vacuolation cell death in lung cancer cells.