Lung cancer growth inhibition and autophagy activation by tetrazole via ERK1/2 up-regulation and mTOR/p70S6K signaling down-regulation

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INTRODUCTION

Lung cancer, a most common clinically diagnosed malignancy worldwide is detected in two forms: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Seymore, 1999). SCLC has been reported to grow rapidly and undergo metastasis/diffusion to distant organs at much faster rate compared to NSCLC (Yang et al., 2009). During the last few decades, several countries including China reported a drastic increase in incidence as well as mortality rate of the lung cancer. Although etiology of lung cancer is not clearly known, reports have found close association of pulmonary cancer with long-term smoking (Bekhit et al., 2004). Among globally diagnosed lung cancer patients ~80% cases account for NSCLC. Clinically it has been demonstrated that there is post-operative recurrence in most of the NSCLC patients undergoing treatment (Bekhit et al., 2004). Patients suffering from advanced stage of NSCLC have very poor prognosis (Shimizu et al., 2004) and therefore effective strategies are needed to control lung cancer development.

Autophagy, a cellular homeostasis process, plays a key role in degradation of dysfunctional/unnecessary contents-proteins or organelles in living beings (Bolt et al., 2012). Autophagy regulates survival response and its over-activation induced by pathogen associated mechanism or due to nutrient starvation becomes fatal for cells as it leads to cell death via a non-apoptotic pathway (Klionsky, 2014). A signalling mechanism identified for autophagy activation in mammalian cells involves mammalian target of rapamycin (mTOR) pathway (Wang et al., 2008). Activation of PI3K/AKT/mTOR signalling has been identified to promote growth, proliferation, and metastasis in different kinds of cancers (Santoni et al., 2013). However, targeting PI3K/AKT/mTOR pathway has been revealed as an appropriate strategy to regulate tumor development (Nazio et al., 2013). Additionally, autophagy activation is also linked to the extracellular signal-regulated kinases 1/2 (ERK1/2) (Hosokawa et al., 2009).

Tetrazole fragment constitutes a promising pharmacophore and is being synthesized in many compounds to improve bioactivity in drug development program (Favata et al., 1998). Flexibility combined with stability on binding to various targets is the main factor which led to advances in tetrazole chemistry (Fukazawa et al., 2002). Another characteristic property that attracts towards tetrazoles as pharmacophores is their ability to act...
as carboxylic functionality mimicking agents. Because of this property tetrzaoles showed effective anti-inflamma-
tory potential (Sharma et al., 2018) and act as analgesics
and antiulcer agents (Tang et al., 2017). Gravacridondiol
is isolated from Ruta graveolens L. that is a perennial herb,
originally native of the Mediterranean region (Tanida,
2011). Formulations of R. graveolens have been used to
inhibit progression or to completely repress the glioma
growth in human brains without causing toxicity (Klion-
sky et al., 2012). In the present study gravacridondiol
tetrzaole (tetrazole) was synthesized and investigated for
lung cancer growth inhibition potential in vitro. Addition-
ally, the mechanism associated with tetrazole mediated
cell death was also explored.

MATERIALS AND METHODS

Cell culture

Beas-2B normal epithelial lung cells, A549 and
NCI-H1819 lung cancer cell lines were procured from
ATCC (Manassas, VA, USA) and grown in DMEM
(HyClone, Logan, UT) mixed with FBS (10%). Medium
was also mixed with penicillin (100 U/mL) and strep-
tomycin (100 mg/mL). The cells were incubated over-
night at 37°C in an incubator under 5% CO2 and 95%
air atmosphere.

Synthesis of gravacridondiol tetrzaole (tetrazole)

R. graveolent plant material was obtained from the
China-Ladakh border and identified by Prof. Zhang Li.
Roots of the plant were dried under shade, chopped,
and then finely powdered in grinders to collect the pow-
der. The powdered plant material was extracted in ethyl
acetate solvent for 48 h, the solvent was decanted and
concentrated in rotary vacuum evaporator to obtain the
extract. The extract was mixed with silica gel to prepare
slurry, which was loaded onto a silica gel column to iso-
late the gravacridondiol compound.

The solution of gravacridondiol in aqueous alco-
hol was treated with 1,3-diido-5,5-dimethylhydantoin
(2 equivalent; DHI) in the dark at 70°C for 12 h. The
crude nitrile compound (2; Scheme 1) was purified by
column chromatography, dried, and then weighed. The
nitrile (2; Scheme 1) obtained was dissolved in DMF and
then reacted with sodium azide (2 equivalent) and iodine
(6 mol%) for 24 h at 60°C to obtain the desired tetrza-
ole (3; Scheme 1). Impure tetrzaole (3; Scheme 1) was
loaded onto a column and purified using ethyl acetate/
petroleum ether (50:50) solvent system. Tetrzaole was
characterized using spectral techniques like 1H NMR,
HRMS and IR spectroscopy.

Viability assay

The cellular density in 96-well plates was adjusted at
2×104 cells/well and grown for overnight in an incuba-
tor at 37°C. Tetrzaole at 1.5, 3, 6 and 12 μM was mixed
with DMEM and cell incubation was performed for 72
h with it. Then, medium from the wells was replaced by
fresh medium free from tetrzaole. Afterwards, MTT
solution (0.5 mg/ml) was poured into each well and
incubation of cells was carried out for 4 more hours
under the same conditions. Discarding of medium from
the wells was followed by an addition of 100 μl DMSO
solubilization solution. Gentle shaking of plates to uni-
formly mix the colour was followed by optical density
measurements, in triplicates, at 570 nm using the mi-
croplate reader (Wellscan MK3, Labsystems, Finland).

Cell cycle analysis

The cells were distributed at 2×104 cells/well density
in 6-well plates and cultured for overnight at 37°C. Ce-
cular distribution in different phases was detected fol-
lowing propidium iodole (Sigma-Aldrich) staining of the
DNA content. Briefly, trypsinization and then overnight
fixing in 70% ethanol alcohol were followed by PBS-wash-
ing of the cells two times. Afterwards, centrifugation
was performed at 235×g and the separated cells were treated
for 15 min with RNase (50 μl) prior to PI (50 μg/ml)
staining for 2 h. The FACSARia II instrument for flow
cytometry and CellQuest7.6.2 software (BD Biosciences,
CA, USA) was employed for the determination of cell
fraction in various cell cycle phases.

Western blotting

The cells were distributed at 2×104 cells/well density
in 6-well plates and incubated with tetrzaole at 6 and 12
μM for 72 h. Exposure to tetrzaole was followed by cell
washing two times with cold-PBS and treatment with
lysis buffer consisting of NP-40 (1%), sodium phos-
phate (5 mM), sodium chloride (150), Tris-HCl (pH 7.5;
20 mM), Na2VO3 (5 mM), PMSF (1 mM) and leupeptin
(10 μg/ml). Lysate formed after 30 min was centri-
fuged for 20 min at 15,000×g to collect the supernatants
in which protein content was estimated by BCA kits.
Samples were loaded in equal amounts (20 μg) to resolve
bands on 12% SDS-PAGE followed by transfer onto the
PVDF-membranes. Membrane incubation was made at
4°C with primary antibodies overnight, followed by PBS/Tween 20 washing for 1 h. Afterwards, incubation
was carried out with the secondary antibodies conjugated
to the goat anti-rabbit IgG for 2 h at room temperature.
Band visualization was made using SignalFire™ ECL
Reagent (Cell Signaling Technology, Inc.) and quantifica-
tion by Image J version 2.0 software (Bio-Rad Laborato-
ries Inc, USA). Antibodies used were against: p-ERK1/2
(dilution 1:1200), LC3 (dilution 1:1200), sequestosome-1
(SQSTM1/p62; dilution 1:2000), ERK1/2 mTOR (dilu-
tion 1:1200), p-mTOR (dilution 1:1200), p70S6K (dilu-
tion 1:2000) and β-actin (dilution 1:1200; all from Cell
Signaling Technology, Inc., Danvers, MA, USA).

Apoptosis assays

Cells were seeded at 2×104 cells/well density in 6-well
plates and incubated with tetrzaole at 12 μM for 72 h.
Cells exposed to tetrzaole were stained as per instruc-
tions of the supplier with Annexin V/PI (BD Bio-
sciences, NJ, USA) for apoptosis detection. Incubation
of A549 and NCI-H1819 cells with Annexin-V/PI was
performed in the dark for 20 min. Then, apoptosis was
observed in cells by a flow cytometer (BD Biosciences,
CA, USA) was employed for the determination of cell
fraction in various cell cycle phases.
RESULTS

Tetrazole suppresses A549 and NCI-H1819 cell viability

Tetrazole was synthesised in two steps from gravacridondiol using 1,3-diiodo-5,5-dimethylhydantoin (DIH) as reagent (Scheme 1). Viability changes by tetrazole at 0.75, 1.5, 3, 6 and 12 μM in A549 and NCI-H1819 cells were measured using MTT assay (Fig. 1). In tetrazole-treated cells a significant ($P<0.05$) suppression in viability was measured in dose-based manner with an increase in the tetrazole concentration. Tetrazole treatment of A549 and NCI-H1819 cells suppressed viabilities to 89% and 86%, respectively at 0.75 μM concentration. Treatment with 12 μM tetrazole suppressed viabilities to 23% and 20%, respectively in A549 and NCI-H1819 cells. Tetrazole treatment did not affect the viability of Beas-2B normal epithelial lung cells (not shown).

Tetrazole leads to A549 and NCI-H1819 cell cycle arrest in G1-phase

Tetrazole exposure at 6 and 12 μM for 72 h was followed by flow cytometric detection of A549 and NCI-H1819 cells for cell cycle changes (Fig. 2A, B). In 12 μM tetrazole exposed A549 cells, G1-phase cell count increased to 71.44±3.98% compared to 43.76±3.14% in control. In NCI-H1819 cells tetrazole exposure at 12 μM raised G1-phase cell population to 75.72±4.52% compared to 43.86±3.61% in the control. Tetrazole exposure caused significant ($P<0.05$) lowering of cell population in S and G2/M phases in A549 and NCI-H1819 cells at 72 h.

Tetrazole promotes LC3-II and lowers SQSTM1/p62 in A549 and NCI-H1819 cells

In tetrazole-treated A549 and NCI-H1819 cells a prominent raise in LC3-II expression was observed at 72 h relative to control (Fig. 3). Tetrazole induced LC3-II expression increase was higher in 12 μM exposed
A549 and NCI-H1819 cells compared to cells exposed to 6 μM. The level of SQSTM1/p62 expression showed a marked lowering in A549 and NCI-H1819 cells on exposure to tetrazole. Lowering of SQSTM1/p62 expression by tetrazole treatment was more prominent in 12 μM treated A549 and NCI-H1819 cells relative to 6 μM exposed cells.

Tetrazole regulates ERK1/2 and mTOR phosphorylation in A549 and NCI-H1819 cells

The p-ERK1/2 level showed a prominent enhancement in A549 and NCI-H1819 cells on exposure to tetrazole at 6 and 12 μM (Fig. 4A). In 12 μM tetrazole treated cells, p-ERK1/2 expression was enhanced to maximum level compared to 6 μM treated A549 and NCI-H1819 cells. Exposure of A549 and NCI-H1819 cells to 6 and 12 μM tetrazole significantly lowered p-mTOR expression at 72 h relative to the control (Fig. 4B). Treatment with tetrazole caused significant (P<0.02) inhibition of p-p70S6K expression in A549 and NCI-H1819 cells at 6 and 12 μM doses.

Tetrazole mediated LC3II/I increase in A549 and NCI-H1819 cells is reversed by U0126

Tetrazole treatment at 12 μM significantly (P<0.05) raised LC3II/I ratio in A549 and NCI-H1819 cells relative to control (Fig. 5). However, exposure to U1026 alleviated tetrazole mediated LC3II/I ratio increase in A549 and NCI-H1819 cells significantly (P<0.02) compared to tetrazole treated cells.

Tetrazole promotes A549 and NCI-H1819 cell apoptosis

Significant (P<0.05) rise in apoptotic cell count was observed in A549 and NCI-H1819 cells on treatment with tetrazole at 12 μM (Fig. 6). Exposure to 3-MA could not increase A549 and NCI-H1819 cell apoptotic percentage when compared to the control cells. Treatment with tetrazole (12 μM) and 3-MA in combination led a significant (P<0.02) elevation in A549 and NCI-H1819 cell apoptotic count relative to tetrazole (12 μM) alone treated cells.

Tetrazole mediated A549 and NCI-H1819 cell apoptosis is elevated by 3-MA

In A549 and NCI-H1819 cells tetrazole treatment promoted caspase-3 cleavage markedly at 12 μM doses relative to control cells (Fig. 7). Exposure to 3-MA could not increase caspase-3 cleavage in A549 and NCI-H1819 cells. However, when A549 and NCI-H1819 cells were treated with tetrazole and 3-MA combination cleaved caspase-3 level showed marked up-regulation relative to tetrazole-treated cells.

DISCUSSION

Chemotherapeutic strategy for cancer has advanced into a novel era wherein molecular pathways are targeted using highly selective drugs that are free from conventional cytotoxicity (Bursch, 2001). Invented tetrazole derivatives were found to be highly active and yielded promising results as anti-cancer agents (Mihaylova et al., 2011). Compounds containing tetrazole rings have been demonstrated to exhibit diversity of biological activities (Pyo et al., 2012). The present study found that tetrazole repressed A549 and NCI-H1819 cancer cells viability depending upon the concentration added. Tetrazole exposed A549 and NCI-H1819 cells were examined by flow cytometry for possible changes in cell cycle progression. It was found that tetrazole exposure induced an arrest of cell cycle in A549 and NCI-H1819 cells in G1-phase. Tetrazole (12 μM) exposure raised A549 and NCI-H1819 cell population from 43% to more than 70% in the G1-phase. Consequently, significant (P<0.05) lowering of cell population in S and

Figure 3. Effect of tetrazole on autophagy markers in A549 and NCI-H1819 cells.

Figure 4. Effect of tetrazole on mTOR/ERK signalling in A549 and NCI-H1819 cells.

Figure 5. Effect of U1026 on tetrazole induced higher LC3II/I ratio.
G2/M phases was found in tetrazole exposed A549 and NCI-H1819 cells. Autophagy is actually a process of homeostasis associated with delivery of various intracellular constituents to be degraded into the lysosomes. Besides several environmental stresses, chemotherapeutics also activate autophagy in tumor cells in different organs (Schmidt-Kittler et al., 2010). Over-activated autophagy acts as another process to eliminate cells in programmed but nonapoptotic way (Kharas et al., 2010). A prominent marker indicating autophagy activation is the expression of LC-3 formed from LC3-I (Dancey et al., 2012). There is also a reduction in p62 level because of its degradation along with other autophagosomal contents during autophagy activation (Cagnol et al., 2010). In the present study tetrazole-treatment of A549 and NCI-H1819 cells led to a prominent raise in LC3-II expression relative to control cells. Moreover, tetrazole mediated LC3-II expression increase was higher in 12 μM exposed A549 and NCI-H1819 cells compared to the cells exposed to 6 μM. Additionally, SQSTM1/p62 level showed a remarkable lowering in A549 and NCI-H1819 cells on exposure to tetrazole. This indicates that tetrazole treatment over-activates autophagy in A549 and NCI-H1819 cells, leading to growth inhibition. Autophagy induction associated with nutrient starvation or other stress factors is regulated mainly by two pathways such as mTOR and ERK1/2 (Popova et al., 2017). Elevation in p-ERK expression in bladder (J82 & 5637 cells) and adrenocortical carcinoma cells has been demonstrated to over-activate autophagy and inhibit tumor growth (Bachar et al., 2004). It is reported that mTORC1 avoids autophagy via deactivation of proteins and induction of mTOR and p70S6Kinase-1 phosphorylation (Natrajan et al., 2010). In the present study p-ERK1/2 level showed a prominent enhancement in A549 and NCI-H1819 cells on exposure to tetrazole at 6 and 12 μM. Exposure of A549 and NCI-H1819 cells to 6 and 12 μM tetrazole significantly lowered p-mTOR expression at 72 h relative to the control. Treatment with tetrazole caused significant (P<0.02) inhibition of p-p70S6K expression in A549 and NCI-H1819 cells at 6 and 12 μM doses. Inhibition of MEK1/MEK2 belonging to MAPK/ERK kinases is achieved in a highly selective manner using U0126 (Hallinan et al., 2002). It is established that U0126 effectively blocks ERK pathway (Meepagala et al., 2005) and because of this A549 and NCI-H1819 cells were exposed to U0126 to confirm tetrazole mediated ERK1/2 activation. In the present study tetrazole treatment significantly (P<0.05) raised LC3II/I ratio in A549 and NCI-H1819 cells relative to control. However, exposure to U0126 alleviated tetrazole mediated LC3II/I ratio increase in A549 and NCI-H1819 cells significantly (P<0.02) compared to tetrazole treated cells. Autophagy mediated cell death has been confirmed previously in carcinoma cells during antineoplastic studies (Pathak et al., 2003). The present study used 3-MA to block tetrazole mediated autophagy induction and investigate any increase in cell apoptosis. A significant

![Figure 6. Effect of 3-MA and tetrazole on apoptosis.](image)

A549 and NCI-H1819 cells were treated with tetrazole at 12 μM and then exposed to 3-MA at 5 μM doses for 72 h. Apoptotic death in A549 and NCI-H1819 cells was detected by Flow cytometry. *P<0.05, **P<0.02 vs. without tetrazole.

![Figure 7. Effect of 3-MA and tetrazole on caspase-3 activation.](image)

A549 and NCI-H1819 cells were treated with tetrazole at 12 μM and then exposed to 3-MA at 5 μM doses for 72 h. Activated caspase-3 was examined in (A) A549 and (B) NCI-H1819 cells by western blotting. *P<0.05, **P<0.02 vs. without tetrazole.
(P<0.05) rise in apoptotic cell count in A549 and NCI-H1819 cells was observed on treatment with tetrazole. Exposure to 3-MA could not increase A549 and NCI-H1819 cell apoptotic percentage when compared to the control cells. Treatment with a combination of tetrazole (12 μM) and 3-MA led to a significant (P<0.02) elevation in A549 and NCI-H1819 cell apoptotic count relative to tetrazole alone treated cells. These finding indicated that tetrazole inhibited A549 and NCI-H1819 cell growth via autophagy activation. In A549 and NCI-H1819 cells tetrazole treatment promoted caspase-3 cleavage markedly at 12 μM doses relative to control cells. Exposure to 3-MA could not increase caspase-3 cleavage in A549 and NCI-H1819 cells. However, when A549 and NCI-H1819 cells were treated with tetrazole and 3-MA combination, cleaved caspase-3 level showed marked up-regulation relative to tetrazole-treated cells.

CONCLUSION

In summary, tetrazole manifested anti-proliferative effect on lung cancer cells via autophagy over-activation and the arrest of cell cycle. It deactivated ERK1/2 signalling and promoted mTOR signaling in A549 and NCI-H1819 cells to regulate cancer proliferation. Thus, tetrazole needs to be studied further as an anti-proliferative agent for the treatment of lung cancer.

Acknowledgements

Not applicable.

Competing interests

The authors declare that there is no conflict of interest to disclose.

Statement of Ethics

The approval for present study was obtained from the Research Ethics Committee, The Third People’s Hospital of Chengdu, Sichuan, China. All the experimental procedures were conducted in accordance with the guidelines issued by National Institute of Health (NIH), USA.

SUMMARY

Treatment with 12 μM tetrazole suppressed viabilities to 23% and 20% in A549 and NCI-H1819 cells, respectively. In tetrazole exposed cells, G1-phase cell count increased significantly compared to the control. Tetrazole-treatment of A549 and NCI-H1819 cells caused a prominent raise in LC3-II and p-ERK1/2 expression. The SQSTM1/p62 level, p-mTOR and p-p70S6K expression was significantly lowered in A549 and NCI-H1819 cells on exposure to tetrazole.

Exposure to U1026 alleviated tetrazole mediated LC3II/I ratio increase in A549 and NCI-H1819 cells significantly (P<0.02) compared to tetrazole treated cells. Treatment with tetrazole and 3-MA in combination led a significant (P<0.02) elevation in A549 and NCI-H1819 cell apoptotic count relative to tetrazole (12 μM) alone treated cells. Tetrazole and 3-MA combination increased cleavage of caspase-3 to a greater extent compared to tetrazole.

Authors’ contributions

Hongyang Zhang conceived and designed the study, Liangjian Zheng, Jun Zhang, Jia Fan, Yuxin He, Tingting Zhan performed experimental work and carried our literature survey. Liwen Rong, Liangjian Zheng and Jun Zhang compiled and analysed the data. Hongyang Zhang and Liangjian Zheng wrote the paper. All the authors approved the paper for publication.

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