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Research paper

Targeting apoptosis by 1,2-diazole through regulation of EGFR, Bcl-2 and CDK-2 mediated signaling pathway in human non-small cell lung carcinoma A549 cells

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ABSTRACT

Lung cancer is the leading cause of cancer deaths worldwide and non-small cell lung carcinoma (NSCLC), a heterogeneous class of tumors, represents approximately 85% of all new lung cancer diagnosis. Conventional treatment options have limited efficacy because most cases are in the advanced stage at the time of diagnosis. The present study evaluates the anti-cancer activity of 1,2-diazole (pyrazole), a natural compound from mangrove plant *Rhizophora apiculata* (R.apiculata) on A549 lung carcinoma cells. In the present study the anti-cancer mechanism of pyrazole, was examined by the expression level of proteins Epidermal growth factor receptor (EGFR), Bcl-2-associated X protein (Bax), B-cell lymphoma-2 (Bcl-2) and Cyclin-dependent kinase-2 (CDK-2) which are commonly associated with the cell signaling pathways that control cell survival and apoptosis, that could facilitate to develop a novel target and effective treatment approach for patients with NSCLC. Pyrazole significantly induced cell cycle arrest and initiated apoptosis through inhibition of downstream components of EGFR tyrosine kinase pathway. Pyrazole disrupts the mitochondrial membrane potential and modulated the protein levels of Bax and Bcl-2 which could probably lead to caspase-3 activation. Furthermore, Pyrazole suppresses the expression of CDK-2 resulting in cell cycle arrest at G1 phase and in the G1-S phase transition. Taken together, the current study provides new insight in to the precise molecular mechanisms responsible for the anti-cancer activity of pyrazole in NSCLC, A549 cells. The study opens an avenue for development of a natural compound as a potential therapeutic agent which could target cell signaling pathways to combat human NSCLC.

1. Introduction

Lung cancer is one of the leading cause of cancer deaths worldwide (Ferlay et al., 2015; Torre et al., 2012). The global burden of cancer continues to remarkably increase which accounts for 8.8 million deaths in 2015 and is expected to rise up to ≈14 million deaths by the year 2030 (Gridelli et al., 2015). Lung cancers are classified into small-cell lung cancer (SCLC) and non-small-cell lung carcinoma (NSCLC), among the lung cancers,85% being NSCLC according to the histological types published by the World Health Organization (WHO) (Langer et al., 2010; Gridelli et al., 2015). The therapeutic strategies for lung cancer treatment include surgery, chemotherapy and radiotherapy. The primary treatment choice of early stage lung cancer is surgery. Unfortunately, 85% of the patients are in the advanced stage at the time of diagnosis and lose the chance for surgery due to lack of symptoms in early NSCLC (Prabhu and Guruvayoorappan, 2013; Li et al., 2016). Conversely, recent advances in the treatment of NSCLC have served as part of a paradigm of “personalized” medicine in oncology. For example, inhibitors that target epidermal growth factor receptor (EGFR) signaling pathways, inducers of apoptosis and regulators of the cell cycle could be novel and effective treatment approaches for lung cancer. Nowadays, drugs from natural sources, rather than synthetic drugs are becoming more popular and provide alternative treatment options for patients with NSCLC (Kim et al., 2015).

Abbreviations: SCLC, small-cell lung cancer; NSCLC, non-small-cell lung carcinoma; WHO, World Health Organization; PVDF, Polyvinylidene difluoride; LDH, Lactate dehydrogenase; Annexin V-FITC, Annexin V-fluorescein isothiocyanate; SD, Standard deviation; EGFR, Epidermal Growth Factor Receptor; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; CDK-2, Cyclin-dependent kinase-2; FOXO1, Forkhead box protein O1

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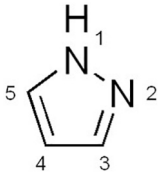


Fig. 1. Structure of 1,2-diazole (pyrazole), a plant alkaloid.

Natural products have long been known as potential sources of therapeutic and effective medicines with, no or less, adverse effects (Prabhu et al., 2013).Plant derived medicines have made large contributions to human health and well-being (Saranraj and Sujitha, 2015).Marine mangrove plants are widely used in medicine to treat a variety of diseases. Among them, *Rhizophora apiculata* (*R. apiculata*) has been screened based on its traditional claim and we have reported that *R. apiculata* exhibits anti-inflammatory, anti-ulcer and anti-tumor properties (Prabhu and Guruvayoorappan, 2012; Prabhu and Guruvayoorappan, 2014).Our earlier studies showed that *R. apiculata* has predominant content of 1,2-diazole (pyrazole) in the crude methanolic extract which was observed to possess anti-inflammatory and nephroprotectant activity (Prabhu et al., 2012; Prabhu et al., 2013). The pyrazole (Fig. 1) are a class of aromatic ring organic compound characterized by the ring structure of 3 carbon atoms and 2 nitrogen atoms that are in adjacent positions. The 1,2-diazole derivatives were first isolated from the seeds of watermelon and are classified as alkaloids (Prabhu and Guruvayoorappan, 2012;Prabhu et al., 2013). Pyrazole derivatives has been reported to be pharmacologically more effective that possess a wide range of biological activities such as antimicrobial, anti-fungal, anti-tubercular, anti-inflammatory, anti-convulsant, anticancer and anti-viral (Ramesh and Bhalgat, 2011; Pluta et al., 2011; Chauhan et al., 2011; Naim et al., 2016). Many pyrazole derivatives has already exist as anti-inflammatory drugs clinically such as phenazone, metamizole, aminopyrine, phenylbutazone, sulfinpyrazone and oxyphenbutazone (Naim et al., 2016). Preliminary studies with pyrazole have shown inhibition of cell proliferation and induced apoptosis in human lung carcinoma A549 cell line. In order to further investigate, the anti-cancer mechanism of pyrazole, we examined the expression level of proteins EGFR, Bax, Bcl-2 and CDK-2 which are commonly associated with the cell signaling pathways that control cell survival and apoptosis, which could facilitate to develop a novel target and effective treatment approach for patients with NSCLC.

2. Materials and methods

2.1. Antibodies, culture media and reagents

Pyrazole (purity-98%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) and Propidium iodide (PI) were purchased from Sigma-Aldrich Chemicals (USA).Nutrient Mixture F-12 Ham Kaighn's Modification (Ham's F-12), fetal bovine serum (FBS), Trypsin Phosphate Versene Glucose (TPVG solution) and Antibiotic Antinomycotic Solution were purchased from Himedia. Rabbit monoclonal primary antibodies such as EGFR(D38B1) XPO Rabbit monoclonal antibodies (mab),CDK-2(78B2) Rabbit mab, β-actin mab and anti-rabbit HRP conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit monoclonal primary antibodiesBcl-2 and secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Desalted Primers such as EGFR, Bcl-2, Bax and β-actin were purchased from Eurofins Genomics India Ltd., Agarose was purchased from GeNei™ (Bangalore, India). Bovine serum albumim (BSA) has purchased from Himedia. Annexin V-fluorescein isothiocyanate/ Propidium Iodide (Annexin V-FITC)/PI kit was purchased from BD Pharmingen, San Diego, CA. All chemicals and solvents used were of the highest purity grade available.

2.2. Cell culture and preparation of drug

Stock cultures of human lung carcinoma (A549) cells procured from National Centre for Cell Science (NCCS), Pune, India, were grown in a monolayer at 37 °C in a 5% CO2 incubator and maintained in Ham's F-12 containing 10% FBS.75 μM solution of pyrazole was prepared in distilled water and diluted in cell culture medium Ham's F-12, as required. The final concentration of Ham's F-12 was minimized to 0.1% and this did not affect cell survival.

2.3. Evaluation of in-vitro cytotoxicity of pyrazole using MTT and LDH leakage assay

MTT and LDH leakage assays were carried out to determine the effect of pyrazole on cell viability in A549 lung carcinoma cells as described earlier (MadanKumar et al., 2014). Briefly, 1×10^5 A549 cells/well were seeded in 96-well plates and incubated at 37 °C in 5% CO2 for 24 h. On 70% confluence, media with different concentrations of pyrazole (5 μM to 500 μM) were added and incubated at 37 °C in 5% CO_2 for 24 h and 48 h. The samples included a 'blank' (medium alone) and 'control (DMSO alone). After incubation, the percentage of growth inhibition (IC_{50}) and LDH leakage (%) were determined. All experiments were performed in triplicate, and the relative cell viability and LDH leakage was expressed as a percentage relative to the untreated control cells. The above experiments were repeated twice for accuracy. % Survival = (OD test-OD control) × 100.

2.4. Reverse transcription-polymerasechain reaction

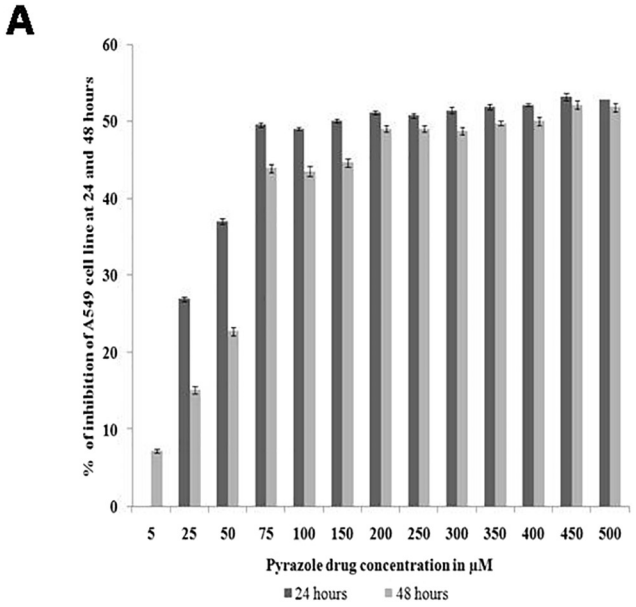
Total RNA was extracted from control and pyrazole treated A549 cells as described previously (Sasaki et al., 2008; MadanKumar et al., 2014). The concentration of the RNA was quantified using biophotometer at optimal density 260 nm (MadanKumar et al., 2014). RNA sample of required calculated volume was transcribed into cDNA by using the one step protocol Thermo Fisher Scientific India Ltd. β-actin was used as loading control for all the experiments. PCR was carried out using the oligonucleotide sequences and amplification conditions as given in Table 1.

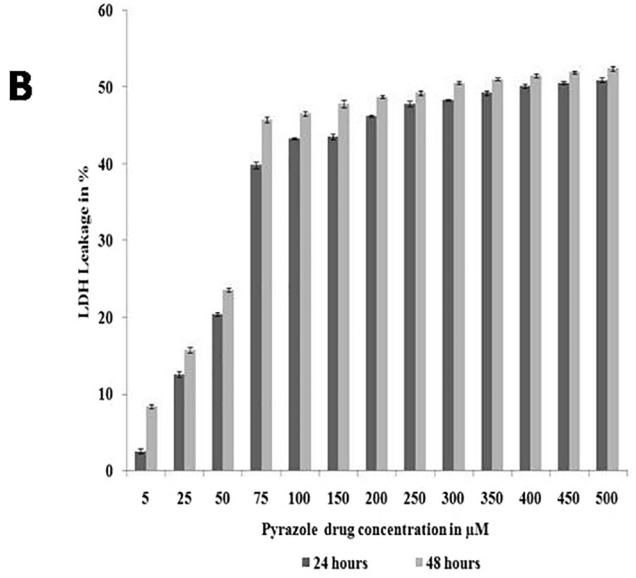
2.5. Western blotting

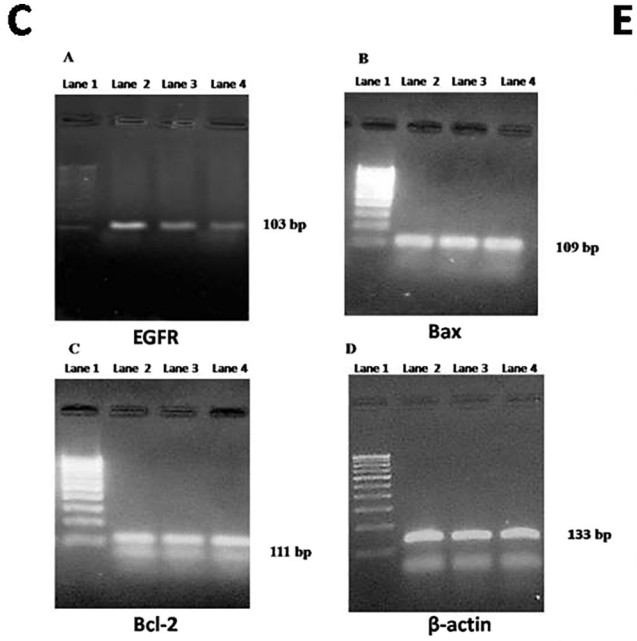
20–40 μg of the protein lysate was separated by SDS-PAGE (10% SDS-acrylamidegel) and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 1:30 h at 100 mA. The membrane was incubated with EGFR Rabbit mab (1:1000 dilution), CDK-2 Rabbit mab (1:1000 dilution), Bcl-2 Rabbit mab (1:1000 dilution) and β-actin mab (1:1000 dilution) from (Cell Signaling Technology) in 5% BSA solution. Following primary antibody incubation, the membranes were incubated with anti-rabbit HRP conjugated secondary antibodies (1:5000 dilution) for 2 h. Finally, the immunoreactive protein complex was detected by using Enhanced chemiluminescent (ECL) system. β-actin served as loading control. The intensities of the protein bands were quantified using Image software

Table 1
List of genes, and their primers with their product size.

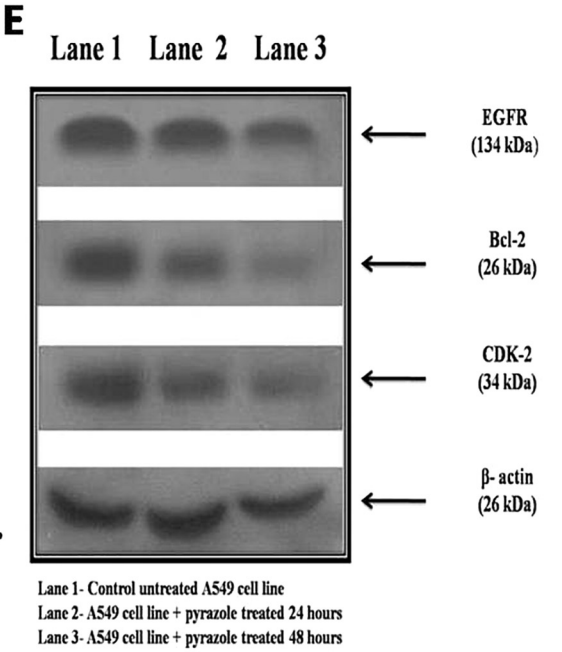
Name of the gene	Primers 5′-3'	Product size (base pair)	Temperature °C
EGFR	**F:**CCTGGTCTGGAAGTACGCAG **R:**GGGAGACTAAAGTCAGACAGTGA	103	60.5
Bax	**F:**AGAACCATCATGGGCTGGAC **R:**CAGTCGCTTCAGTGACTCGG	109	59.2
Bcl-2	**F:**GGATCCAGGATAACGGAGGC **R:**GGGCCAAACTGAGCAGAGTC	111	61.4





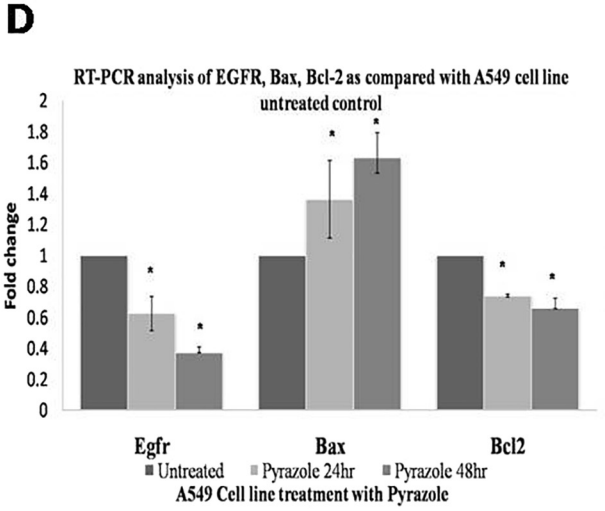


Lane 1- DNA Ladder; Lane 2- Control A549 cell line; Lane 3-A549 cell line + pyrazole treated 24 hours; Lane 4- A549 cell line + pyrazole treated 48 hours



Lane 1- Control untreated A549 cell line
Lane 2- A549 cell line + pyrazole treated 24 hours
Lane 3- A549 cell line + pyrazole treated 48 hours

Western Blot analysis of EGFR, Bcl-2, CDK-2 and β- actin as fold changes as compared with A549 cell line untreated control



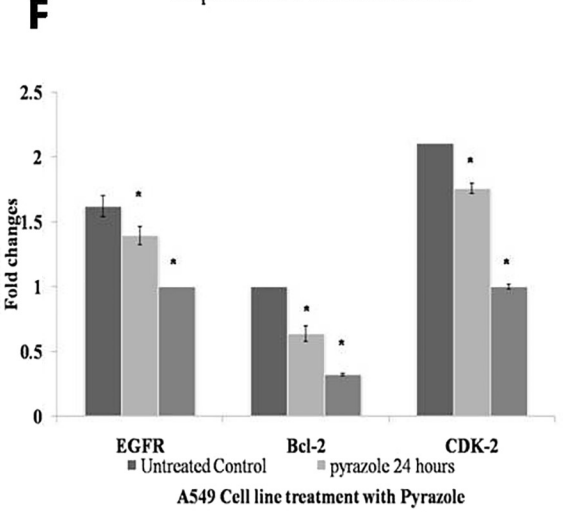


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Fig. 2. In-vitro cytotoxicity of pyrazole in cultured A549 cells using MTT and LDH assay. 2A) Cytotoxic effect of various concentrations of pyrazole on A549 cells: Dose dependent cytotoxic of pyrazole on A549 cells are represented in the above graph. Cells were cultured in 96-well plate and treated with different doses of pyrazole (5–500 μM) for 24 and 48 h. The cell viability was analyzed by MTT assay. A549 cells were sensitive to pyrazole and the IC_{50} at 24 h and 48 h was 75 μM. All the experiments were done in triplicate. 2B) Effect of various concentrations of pyrazole on LDH release by A549 cells: Dose dependent cytotoxicity of pyrazole on A549 cells showing LDH release are represented in the above graph. Pyrazole significantly reduced the viability of A549 cells with increased release of LDH into the medium in a dose and time dependent manner at 24 h and 48 h. 2C) RT-PCR analysis of the expression: (A) EGFR (B) Bax (C) Bcl-2 (D) β-actin are presented as compared with untreated control cells. 2D) Densitometric values of mRNA expression: The densitometric values of mRNA expression of EGFR, Bax and Bcl-2 normalized with β-actin are presented as compared with untreated control cells. Values are expressed as mean ± SD (n = 3). Hypothesis testing method included One-way ANOVA followed by LSD. 2E) Effects of pyrazole on the expressions of EGFR, Bcl-2, CDK-2 and β-actin in A549 cells. A549 cells were treated with at 75 μM concentrations of pyrazole and the expressions of EGFR, Bcl-2, CDK-2 and β-actin were detected by western blot as compared with untreated control cells. Lane 1- Untreated control cells, Lane 2- pyrazole treated cells (24 h), Lane 3- Pyrazole treated cells (48 h). 2F) Densitometric values of the expression of EGFR, Bcl-2 and CDK-2 are resented as compared with untreated control cells. Values are expressed as mean ± SD (n = 3). Hypothesis testing method included One-way ANOVA followed by LSD. Statistical significance at $p < 0.05$. Comparisons are made with untreated control cells. Lane 1- Untreated control cells, Lane 2 – Pyrazole treated cells (24 h), Lane 3- Pyrazole treated cells (48 h). Values are expressed as mean ± SD. Value is significantly different from untreated control ($^*p < 0.05$).

(NIH, Bethesda, USA).Experiments were repeated in triplicate (Lowry et al., 1951; Subramaniya et al., 2011; MadanKumar et al., 2014).

2.6. Apoptotic cell determination by annexin V/PI staining assay using flow cytometry

The A549 cells were seeded at 10^5 cells/mL per well in six well plates at 37 °C in a humidified atmosphere containing 5% CO_2. Then, A549 cells were harvested after treatment with different concentrations of pyrazole (25 μM, 50 μM and 75 μM, respectively) for 48 h and the apoptosis was assessed with flow cytometry using Annexin V-fluorescein isothiocyanate/ Propidium Iodide (Annexin V-FITC)/PI kit (BD Pharmingen, San Diego, CA) according to the manufacturer's protocol and analyzed using fluorescence microscope and FACS calibur flow cytometer (BD FACS Aria Cell Sorter flow cytometry, BD Biosciences). The cells in the early stages of apoptosis were Annexin V positive and PI negative, whereas the cells in the late stages of apoptosis were both Annexin V and PI positive (Koopman et al., 1994; Perumal et al., 2017).

2.7. Statistical analysis

SPSS11.5 was used for statistical analysis. Results were representative of three independent experiments unless stated otherwise. Values were presented as the mean ± standard deviation (SD). One-way Analysis of Variance (ANOVA) test was used to analyze significance between groups. The least significant difference (LSD) method of multiple comparisons with parental and control group was applied when the probability for ANOVA was statistically significant. Statistical significance was determined at a $p < 0.05$ level.

3. Results

3.1. Pyrazole induces cytotoxicity in A549 cells

In order to evaluate the cytotoxicity of pyrazole, A549 cells were exposed to increasing concentrations of pyrazole for 24 h and 48 h. Cell viability and LDH leakage were measured by MTT and LDH leakage assay, respectively. As shown in Fig. 2A and B, pyrazole significantly reduced the A549 cell viability with increased release of LDH into the medium in a dose and time dependent manner at 24 h and 48 h, and the estimated IC_{50}value of pyrazole was 75 μM. These results shows that A549 cells were sensitive to pyrazole, indicating that pyrazole induce cytotoxicity of A549 cells.

3.2. Effect of pyrazole on EGFR, Bcl-2 and Bax mRNA and protein expression associated with apoptosis in A549 cells

To investigate the mechanism of pyrazole mediated apoptosis, some candidate signaling molecules were studied. Expression of genes associated with apoptosis was studied by RT-PCR in A549 lung cancer cells. The mRNA expression of EGFR was examined by RT-PCR which shows a significant difference between the pyrazole treated and untreated control cells ($p < 0.05$). The relative level of EGFR mRNA expression in the A549 cells was significantly downregulated by 2.4-fold in 24 and 48 h treatments, when compared with that in the A549 untreated control cells as shown in Fig. 2c. The Bcl-2 mRNA expression was significantly down regulated in pyrazole treated A549 cells and was constitutively and strongly expressed in untreated control A549 cells. Bax mRNA expression was significantly upregulated in pyrazole treated A549 cells in a time dependent manner when compared with untreated control A549 cells as shown in Fig. 2C&D.

To explore the apoptosis inducing mechanistic action of pyrazole in A549 cells, the expression levels of key molecules of EGFR tyrosine kinase pathway and apoptotic proteins were determined by western blotting. The expression levels of EGFR- tyrosine kinase and anti-apoptotic Bcl-2 were significantly decreased in treated A549 cells (24 h

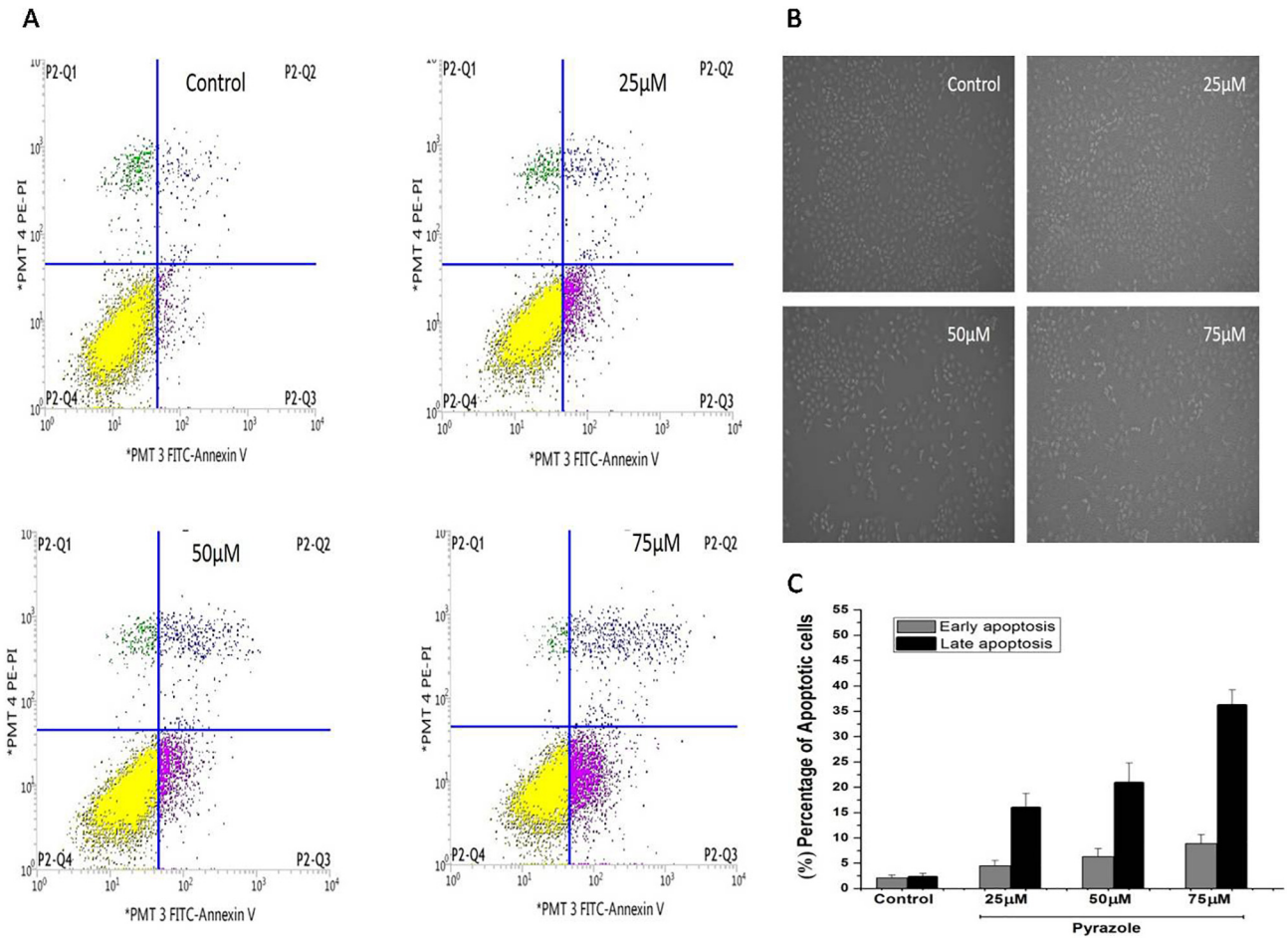

Fig. 3. Effect of pyrazole on apoptosis in A549 cells. (A) Representative dot plots of Annexin V/PI staining. A549 cells were treated with indicated concentrations of pyrazole (25 μM, 50 μM and 75 μM) for 48 h, and stained with Annexin V-FITC/PI solutions according to the manufacturer's manual and detected using flow cytometry. (B) After treatment with pyrazole (25 μM, 50 μM and 75 μM) for 48 h, A549 cells were visualized using phase contrast microscopy. (C) Bar graph representing percentage of early and late apoptotic cells. All experiments were performed in triplicate.

and 48 h),when compared with untreated A549 control cells. Treatment with pyrazole significantly down regulates the expression of CDK-2 in A549 cells in both 24 h and 48 h treatments when compared with the untreated control A549 cells.

3.3. Pyrazole promotes apoptosis in A549 cells

In order to investigate whether the growth-inhibitory effect is related to the induction of apoptosis, A549 cells were treated with pyrazole 25 μM, 50 μM and 75 μM for 48 h. Further confirmation of apoptosis induced by pyrazole was analyzed using flow cytometry based on Annexin V-fluorescein isothiocyanate/ Propidium Iodide (Annexin V-FITC)/PI kit (BD Pharmingen, San Diego, CA). The results of flow cytometry analysis (Fig. 3B,C) show that, the apoptosis in A549 cells was remarkably induced after treatment with pyrazole for 48 h, and treatment of A549 cells with pyrazole at concentrations of 25 μM, 50 μM and 75 μM, resulted in a dose-dependent increase in the numbers of early apoptotic cells (4.54 ± 1.03, 6.33 ± 1.58 and 8.90 ± 1.75), and respectively late apoptotic cells (15.95 ± 2.86, 20.88 ± 3.92 and 36.18 ± 3.12). These data suggested that the induction of apoptosis, at least partly, accounted for the inhibition of A549 cells.

4. Discussion

Lung cancer is the most common form of cancer and leading cause of cancer deaths among both men and women Jemal et al., 2002; Schiller et al., 2002; Kari et al., 2003; Sasaki et al., 2008). NSCLC which comprises of 75% of lung cancer which are complicated to treat due to poorly understood pathological mechanisms (Bethune et al., 2010). Recent advances in understanding of cell signaling pathways that control cell survival would assist to develop novel and effective treatment approaches. Nowadays, drugs from herbal sources, rather than synthetic drugs are becoming more popular and provide alternative treatment options for NSCLC patients (Kim et al., 2015). Possible targets can be evaluated by comparing the cancer cells individual protein amount with those in normal cells. Therefore, proteins that are abundant in cancer cells which regulate cell survival, signaling and progression could be better targets. As example, EGFR which is expressed at high levels on the surface of some cancer cells has been recognized as an effective anti-cancer target (Kari et al., 2003).

EGFR, a receptor tyrosine kinase, is frequently overexpressed in NSCLC. These receptors are involves in tumor cell survival and activated phosphorylation of EGFR results in the phosphorylation of some downstream proteins that cause cell proliferation, invasion, metastasis and inhibition of apoptosis (Lee, 2006). It has been reported that, overexpression of EGFR or mutations in intracellular EGFR have been observed in 43–89% of cases in patients with NSCLC (Gupta et al., 2009).Whereas, similar studies reported that, one quarter of NSCLC had mutations in the EGFR tyrosine kinase domain and these were associated with increased receptor expression in 75% of cases (Grandis and Sok, 2004; Suzuki et al., 2005; Shigematsu and Gazdar, 2006; Merrick et al., 2006; Singh et al., 2016). The EGFR signaling pathway could be inhibited either by blocking the extracellular ligand binding domain with the use of anti-EGFR antibodies or small molecules that restrain

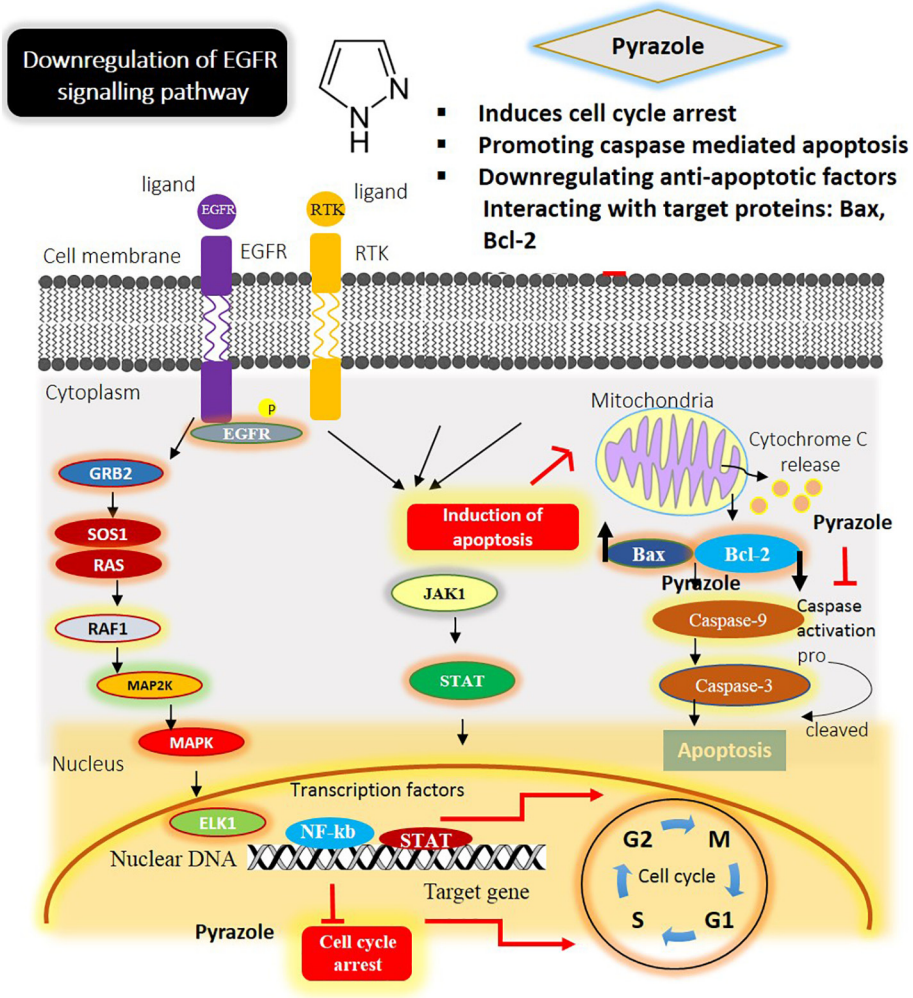

Fig. 4. Proposed mechanism of action of pyrazole in triggering apoptosis through regulation of EGFR, Bcl-2 and CDK-2 mediated signaling pathway in human NSCLC A549 cells. The pathways mediating downstream effects of EGFR have been well studied and proposed mechanism of action in triggering apoptosis by pyrazole could be by three major cell signaling pathways. The first pathway involves RAS-RAF-MAPK pathway, where phosphorylated EGFR recruits the guanine-nucleotide exchange factor via the GRB2 and Shcadapter proteins, activating RAS and subsequently stimulating RAF and the MAP kinase pathway to affect cell proliferation. The second pathway involves PI3K/AKT pathway, which activates the major cellular survival and anti-apoptosis signals via activating nuclear transcription factors such as NFKB. The third pathway involves JAK/STAT pathway which is also implicated in activating transcription of genes associated with cell survival. Targeting BCL-2 is an attractive and rational strategy because BCL-2 gene mutations and BCL-2 protein over expression are clearly associated with lung cancers. Pyrazole promotes down regulation of Bcl-2 protein that triggers the activation of procaspase 9, that in turn activates caspase 3, resulting in cell apoptosis.

the EGFR tyrosine kinase (Yan and Beckman, 2005). The drugs such as poziotinib, gefitinib, erlotinib, canertinib and lapatinib have been approved and are being used as tyrosine kinase inhibitors to treat NSCLC. The tyrosine kinase inhibitor gefitinib, a synthetic anilinoquinazoline which target EGFR, has been reported to have advantageous effect among NSCLC patients (Ono et al., 2004; Kijima et al., 2007; Jackman et al., 2009; Takeda and Nakagawa, 2015).

In the present study, we have reported that pyrazole down regulated both the mRNA and protein levels of EGFR expression in A549 cells. Furthermore the results were supported by apoptosis analysis where A549 cells treated with pyrazole at various concentrations showed significant induction of apoptosis in A549 cells. Therefore, the induction of apoptosis at least partly accounted for the growth inhibition of A549 cells. The outcome shown that, pyrazole could influence the expression of EGFR in lung cancer cells at the transcriptional level and also suggested that, EGFR could be a promising target for NSCLC (Ono et al., 2004; Kijima et al., 2007).To our knowledge, this is the first report that pyrazole down-regulates the expression of EGFR in A549 cells and as a result, EGFR protein expression could be a predictor of successful EGFR-targeted therapy.

Bcl-2 proteins serves as the primary regulators of apoptotic signaling pathways and are classified into members of anti-apoptotic and pro-apoptotic groups (Adams and Cory, 2007; Youle and Strasser, 2008).The tumor cells survives under adverse stress conditions and restores apoptotic pathways by their increased ratio of anti-apoptotic to pro-apoptotic Bcl-2 proteins (Stephen, 2005; Kelly and Strasser, 2011). Therefore by regulating this anti-apoptotic Bcl-2 proteins could be a promising approach for cancer treatment by activating apoptotic pathway (Lindsten et al., 2000). The activation of Bax in tumor cells could enhance effective therapeutic approach in cancer since these Bax proteins are expressed in all cancer cells but their action has been frequently inactivated by anti-apoptotic Bcl-2 proteins (Wei et al., 2001; Vogler et al., 2009). Therefore activation of pro-apoptotic Bax and concurrently inhibiting anti-apoptotic Bcl-2 protein function could bring an effective cancer therapeutic approach. Therefore, in this study, we have shown the modulation of expression of Bax and Bcl-2 in A459 cell line by pyrazole in a dose dependent manner.A549 cells have a high level of Bcl-2 mRNA and a low level of Bax mRNA. Pyrazole significantly down regulated the expression of Bcl-2 mRNA and protein, up-regulated the expression of Bax mRNA and the ratio of Bcl-2 /Bax were reduced, which probably is one of the pro-apoptotic molecular mechanisms of pyrazole in inhibiting the growth of NSCLC. Pyrazole inhibits the growth of NSCLC cells by inducing cancer cell apoptosis via the regulation of the Bcl-2 family thus activating caspase-3 and 9, which may, in part, explain its anti-cancer activity. Therefore, our study has revealed that pyrazole could activate Bax and induce Bax-dependent apoptosis.

Alternation in the regulation of cell cycle could be the future of human cancers that includes CDKs (serine/threonine kinases) that depends on a regulatory subunit - a cyclin (Hunter and Pines, 1994; Sherr, 1994; Morgan, 1995). These cyclins controls cell proliferation by forming complex with different CDKs and the cell cycle transition is coordinated by CDKs integrate mitogenic and growth inhibitory signals (Hartwell, 1992; Sherr and Roberts, 1999; Manning et al., 2002).Cyclin

E/CDK-2 plays a critical role in the G1 phase as well as in G1-S phase transition and regulate the apoptotic response to DNA damage via phosphorylation of Forkhead box protein O1 (FOXO1), which plays an important function in regulation of cell cycle progression (Hinds et al., 1992; Okuda et al., 2000; Massagué, 2004; Lu and Huang, 2011). Many drugs can induce apoptosis of cancer cells through G1 arrest in cancer cells through downregulation of CDK-2 (Li et al., 2014). Flavopiridol, a flavonoid derived from rohitukine plants, docetaxel, SNS-032, ZK-304709, PD-0332991 and ZK-304709 are second generation drugs with therapeutic intervention based on CDK inhibition has been reported for its attractive strategy to treat cancer patients. Likewise, targeting the functions of CDK-2 in DNA replication and S phase progression provides a window of intervention for cancer therapeutics (Nielsen et al., 1996; Wang et al., 1996; Sakaguchi et al., 1998; Motwani et al., 2003; Canavese et al., 2012). In this study, we had reported that pyrazole down regulated CDK-2 expression and induced apoptosis in A549 lung cancer cells in a dose dependent manner. A549 lung cancer cells have a high level of CDK2 expression where pyrazole significantly down-regulated the gene expression of CDK-2 in a time dependent manner, which might be one of the molecular mechanisms of pyrazole in inhibiting the growth of A549 cells by disrupting DNA replication and S phase progression leading to G1 arrest that lead to apoptosis. Therefore, our study revealed that pyrazole could activate cell cycle arrest and induce apoptosis of lung cancer cell through down regulation of CDK-2 in A459 cells. Proposed mechanism of action of pyrazole in triggering apoptosis through regulation of EGFR, Bcl-2 and CDK-2 mediated signaling pathway in A549 cells is shown in Fig. 4.

In summary, the present study is the first of its kind to report the anti-cancer activity possessed by the natural compound pyrazole which exhibits the inhibition of the growth of A549 cells. In A549 cells, pyrazole induced cell cycle arrest and apoptosis through inhibition of downstream components of the EGFR tyrosine kinase pathway and mitochondrial membrane permeability mediated by Bax and Bcl-2, probably leading to caspase-3 activation. Furthermore, CDK-2 inhibition modulates cell cycle arrest at G1 phase and in the G1-S phase transition which regulates the apoptosis. Therefore, the present study greatly contributes to understanding the molecular mechanism responsible for the anti-cancer activity of pyrazole in A549 lung cancer cells mediated via blockade of EGFR signaling pathway. The finding of the present study also opens an avenue for development of natural compounds as potential therapeutic agents that target EGFR signaling pathway to combat human NSCLC.

Conflicts of interest

The authors have no conflict of interest to declare.

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