Tropical Journal of Pharmaceutical Research November 2020; 19 (11): 2343-2348
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i11.14

Original Research Article

Pyrimidine-thioindole inhibits gastric cancer cell proliferation via up-regulation of expression of tumor suppressor miR-145

Rong Zhang[1], Chunhe Yao[2]*
[1]Department of Gastroenterology, [2]Department of General Surgery, Xianyang Hospital of Yan'an University, Xianyang, Shaanxi 712000, China

For correspondence:* ***Email:* *zhangrzt@126.com;* ***Tel:*** *0086-029-3335 1603*

Sent for review: 15 June 2020 *Revised accepted: 25 October 2020*

Abstract

Purpose: *To investigate the effect of pyrimidine-thioindole on gastric cancer proliferation and the underlying mechanism of action.*
Methods: *Cell viability and apoptosis were determined using MTT assay and Annexin V/PI assay, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) was used for the determination of expression levels of miR-145, while protein expression levels were assayed by western blotting.*
Results: *Pyrimidine-thioindole treatment significantly inhibited the proliferation of AGS and SNU-5 cells ($p < 0.05$), but had no effect on the viability of GES-1 cells. Exposure to pyrimidine-thioindole at doses of 8 and 10 μM significantly enhanced the apoptosis of AGS and SNU-5 cells ($p < 0.05$). Pyrimidine-thioindole exposure markedly increased the proportions of AGS and SNU-5 cells in G1 phase ($p < 0.05$). In AGS and SNU-5 cell lines, pyrimidine-thioindole exposure at doses of 8 and 10 μM significantly upregulated the expression of miR-145, with higher enhancement of miR-145 expression in AGS cells than in SNU-5 cells. Moreover, pyrimidine-thioindole downregulated the expressions of MMP-2, MMP-9, c-Myc, p-PI3K and p-AKT in AGS and SNU-5 cells. Pyrimidine-thioindole treatment enhanced the expression of p21 in AGS and SNU-5 cells, relative to untreated cells ($p < 0.05$).*
Conclusion: *These results suggest that pyrimidine-thioindole activates apoptotic signaling pathway, leading to reduction in cell proliferation and arrest of cell cycle. Moreover, it de-activates PI3K/AKT pathway and promotes miR-145 expression in AGS and SNU-5 cells. Thus, pyrimidine-thioindole has therapeutic significance for the management of gastric cancer.*

Keywords: *Gastric cancer, Pyrimidine, Indole, Apoptosis, Cell proliferation*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

The generation of lead compounds in drug discovery begins with identification of molecular entities using literature survey programs [1]. Scrutiny based on Hit-to-Clinical Pairs led to the identification of about 45 % of clinically important candidates from recognized starting materials between 2016 - 2017 [2]. Approximately 30 % of compounds discovered using Hit-to-Clinical Pairs concept are useful in the field of oncology [2]. This indicates the

importance of novel anticancer agents in multidrug resistance therapy [2]. The pyrimidine ring, an essential component of the genetic material, has medicinal significance, and is of great interest for clinicians involved in drug discovery [3]. The pyrimidine moiety serves as pharmacophore in small inhibitor molecules with therapeutic potential for several diseases/disorders [4]. Moreover, compounds containing pyrimidine ring also act as antimicrobial, anti-inflammatory and analgesic agents [5].

Molecular frameworks consisting of pyrimidine ring kill tumor cells via several mechanisms such as tyrosine kinase inhibition, deacetylation of histones, and regulation of autotaxins and heat shock proteins [6]. Pazopanib B, a small molecule derived from pyrimidine, was approved by FDA in 2009 as an anti-angiogenetic compound which acts via inhibition of tyrosine kinase receptor [7]. Another molecule containing pyrimidine, CEP-11981 C has been reported as cytotoxic agent with good anti-angiogenetic properties [8]. Indole is a major pharmacophore present in compounds with anti-cancer, anti-HIV anti-inflammatory and oxidant-quenching properties [9]. Gastric cancer is third highest cause of tumor-related deaths worldwide [10].

The poor prognosis of gastric cancer requires effective treatment especially for patients diagnosed with advanced stage of the disease [11]. Clinicians consider the rapid metastasis of tumor cells in gastric carcinoma patients as a major limitation to available treatments [12]. MicroRNAs (MiRNAs) are generally comprised of 19 - 25 nucleotides, and they influence genetic translation via regulation of mRNA expression [13]. The miRNAs are involved in proliferation as well as death of cells [14]. A study has shown that pre-cancerous lesions extracted from colorectal tissues express lower levels of miRNAs [14]. The pathogenesis of gastric cancer has been linked to down-regulation of the expressions of miRNAs [15]. Elevation of miR-145 expression in tumor cells leads to suppression of Myc proto-oncogene protein (c-Myc) [16]. Moreover, miR-145 expression targets gastric cancer growth through down-regulation of MYO6 expression [16].

The current study investigated the effect of pyrimidine-thioindole (Figure 1) on gastric cancer cell proliferation, and the mechanism involved.

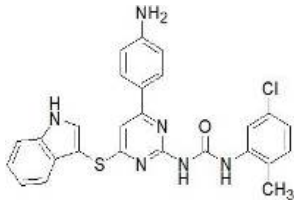

Figure 1: Chemical structure of pyrimidine-thioindole

EXPERIMENTAL

Cell culture

Two gastric carcinoma cell lines i.e. AGS and SNU-5, and normal cell line (GES-1) were supplied by Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in DME medium mixed with 10 % FBS, 1 % penicillin and 1 % streptomycin in a 5 % CO_2 incubator at 37˚C for 24 h.

MTT assay

The AGS, GES-1 and SNU-5 cells were seeded in 96-well plates, each at a density of 2 x 10^5 cells/well, and exposed to pyrimidine-thioindole at doses of 2, 4, 6, 8, 10, 12 and 14 µM for 24 h. Thereafter, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5 mg/mL) was added to each well, followed by incubation for 4 h. The medium was decanted from each well, and the resultant formazan crystals were solubilized in DMSO (120 µL), and the absorbance of each well was read at 560 nm in a multi-well spectrophotometer, after shaking the plate for 10 min.

Determination of apoptosis

The AGS and SNU-5 cells were cultured in 6-well plates for 24 h, each at a density of 1.5 x 10^5 cells per well, with pyrimidine-thioindole at doses of 8 and 10 µM for 48 h. Then, the cells were rinsed twice with cold PBS, followed by resuspending in 300 µL of binding buffer. The cells were then stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 20 min in the dark at room temperature. Fluorescence measurement was done using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) linked with argon laser (488 nm). Cell apoptosis was analyzed using FACScan software (version 6.0; BD Biosciences).

Cell cycle analysis

Treatment of AGS and SNU-5 cells in 6-well plates (2 x 10^6 cells/per well) with pyrimidine-

thioindole at doses of 8 and 10 µM for 24 h was followed by washing in PBS. Then, the cells were fixed overnight in 70 % methyl alcohol at -30 ˚C. Then, Tris-HCl buffer, pH 7.4 containing 1 % RNase A was added to the cells. This was followed by staining with PI (5 mg/mL) prior to determination of DNA content distribution with flow cytometry.

Western blot analysis

The AGS and SNU-5 cells were treated with pyrimidine-thioindole at doses of 8 and 10 µM, followed by incubation for 24 h. Thereafter, the cells were lysed by treatment with 40 mM Tris-HCl buffer, pH 7.4 containing 150 mM sodium chloride, 1 % Triton X-100, and protease inhibitor. The resultant lysates were centrifuged at 12000 g for 30 min at 4 ºC. The protein contents of the supernatants were determined using BCA method. Then, equal amounts of proteins were subjected to 10 – 12 % SDS-PAGE, followed by transfer to PVD membranes previously blocked by incubation with 5 % skimmed milk in TBS and Tween-20 (0.1%). The membranes were probed overnight at 4 ºC with antibodies against cMyc, p-AKT, PI3K, P21, MMP-2, MMP-9 and GAPDH (Cell Signaling Technology, Inc.). Thereafter, the blots were incubated at room temperature for 2 h with horseradish peroxidase-conjugated secondary antibody, followed by washing with 1X PBST. The resultant protein bands were visualized using SignalFire™ Plus ECL system, and quantified with Image J (version 2.0) software.

RT-qPCR

Total RNA was extracted from AGS and SNU-5 cells exposed to pyrimidine-thioindole at doses of 8 and 10 µM d for 24 h, using RNAiso Plus (Dalian, China) reagent. Then, 30 µg of RNA was used for synthesis of first-strand cDNA using the ProSTARt First Strand RT-PCR kit. Quantitative-PCR was carried out with SYBR® Premix Ex Taq™ II kit in accordance with the manual protocol. The primer sequences employed were:

miR-145: forward: 5'-GTC CAG TTT TCC CAG GAA TCC CT-3', reverse: 5'-GCT GTC AAC GAT ACG CTA CCT A-3'.

The amplification conditions used for qPCR consisted of 95 ˚C for 3 min, 39 cycles at 94 ˚C for 25 sec, 52 ˚C for 25 sec, 72 ˚C for 26 sec, and 72 ˚C for 5 min. The relative levels of gene expressions were determined using 2-ΔΔCq method.

Statistical analysis

Data presented are mean ± standard deviation of triplicate measurements. Statistical analysis of data was done with SPSS software (version 17.0; Inc., Chicago, IL, USA). Differences amongst groups were determined using One-Way Analysis of Variance (ANOVA) and Tukey's post-hoc test. Values of $p < 0.05$ were taken as statistically significant.

RESULTS

Cytotoxic effect of pyrimidine-thioindole on AGS and SNU-5 cell lines

The cytotoxicity of pyrimidine-thioindole on AGS and SNU-5 cells was evident in significant suppression of the viabilities of these cells (Figure 2). However, pyrimidine-thioindole treatment had no effect on the viability of GES-1 cells. Exposure to pyrimidine-thioindole at doses of 2, 4, 6, 8, 10, 12 and 14 µM reduced the viability of AGS cells to 91, 78, 61, 47, 38, 28 and 22 %, respectively. For SNU-5 cells, pyrimidine-thioindole treatment at doses of 2, 4, 6, 8, 10, 12 and 14 µM suppressed viability to 93, 80, 65, 51, 42, 30 and 24 %, respectively.

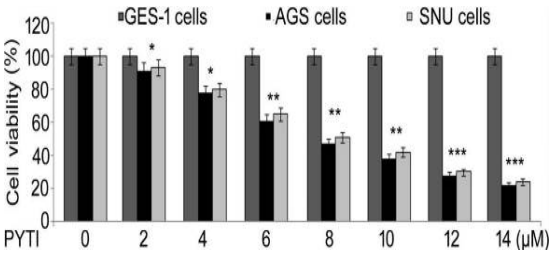

Figure 2: Effect of pyrimidine-thioindole on the viabilities of GES-1, AGS and SNU-5 cells. Treatment of GES-1, AGS and SNU-5 cells with pyrimidine-thioindole at doses of 2, 4, 6, 8, 10, 12 and 14 µM was followed by MTT assay. *$P < 0.05$; ***$p < 0.01$, vs. control cells

Pyrimidine-thioindole induced apoptosis in AGS and SNU-5 cells

Exposure to pyrimidine-thioindole at doses of 8 and 10 µM significantly ($p < 0.05$) enhanced apoptosis of AGS and SNU-5 cell (Figure 3). Pyrimidine-thioindole treatment at doses of 8 and 10 µM raised apoptosis to 43.68 and 57.38 %, respectively in AGS cells at 24 h. In SNU-5 cells, apoptosis reached 38.41 and 51.63 %, respectively on exposure to 8 and 10 µM pyrimidine-thioindole.

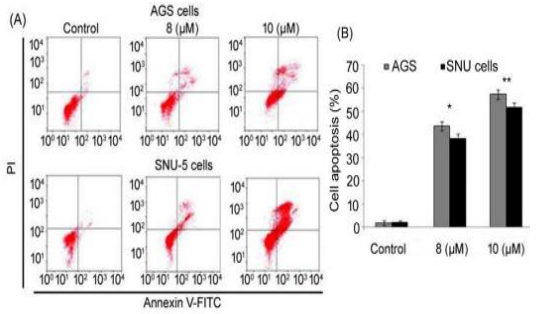

Figure 3: Effect of pyrimidine-thioindole on apoptosis of AGS and SNU-5 cells. (A) Apoptosis of AGS and SNU-5 cells at 24 h of exposure to 8 and 10 μM pyrimidine-thioindole, as analyzed using flow cytometry. (B) Percentage apoptosis in AGS and SNU-5 cells. *$P < 0.05$; **$p < 0.02$, vs. control cells

Pyrimidine-thioindole caused cell cycle arrest in AGS and SNU-5 cells

As shown in Figure 4, pyrimidine-thioindole exposure significantly elevated the proportions of AGS and SNU-5 cells in G1 phase, and suppressed the proportions of AGS and SNU-5 cells in S and G2/M phases ($p < 0.05$). On exposure to pyrimidine-thioindole at doses of 8 and 10 μM, the G1 phase fraction of AGS cells increased to 68.87 and 76.54 %, respectively, while in SNU-5 cells, the G1 phase cells increased to 66.78 and 72.32 %, respectively.

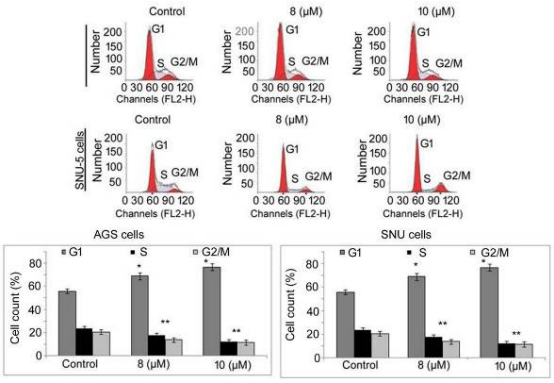

Figure 4: Effect of pyrimidine-thioindole on the cell cycle. (A) Exposure of AGS and SNU-5 cells to pyrimidine-thioindole at doses of 8 and 10 μM for 24 h was followed by DNA content analysis using flow cytometry. (B) DNA content distribution. *$P < 0.05$; **$p < 0.02$, vs. control cells

Pyrimidine-thioindole elevated miR-145 expression in AGS and SNU-5 cells

Exposure of AGS and SNU-5 cells to pyrimidine-thioindole at doses of 8 and 10 μM led to marked and dose-dependent elevations in the expression of miR-145 (Figure 5). Pyrimidine-thioindole treatment caused more enhancement in miR-145 expression in AGS cells than in SNU-5 cells.

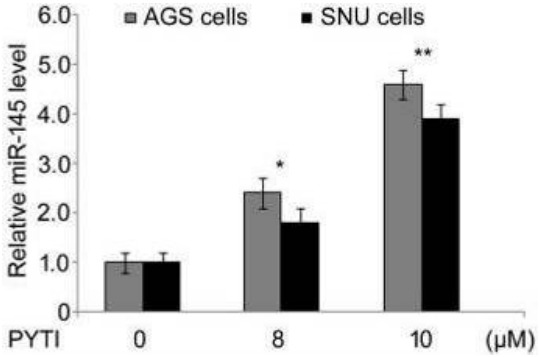

Figure 5: Effect of pyrimidine-thioindole on mRNA expression levels of miR-145 in AGS and SNU-5 cells. *$P < 0.05$; ***$p < 0.02$, vs. control cells

Pyrimidine-thioindole influenced PI3K/AKT pathway

Exposure of AGS and SNU-5 cells to pyrimidine-thioindole caused marked downregulations in the protein expression levels of MMP-2, MMP-9, c-Myc, p-PI3K and p-AKT (Figure 6). Reductions in expressions were higher in pyrimidine-thioindole-treated AGS cells than in SNU-5 cells.

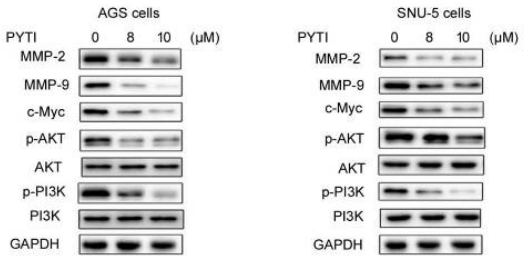

Figure 6: Effect of pyrimidine-thioindole on activation of PI3K/AKT. Expression levels of PI3K, p-AKT, c-Myc, p-PI3K, MMP2 and MMP9 in GES-1, AGS and SNU-5 cells treated with pyrimidine-thioindole at doses of 8 and 10 μM, as assayed using western blotting

DISCUSSION

Each year, clinicians report more than one million cases of gastric cancer which impacts adversely on peoples' health globally [17]. Most oncologists believe that tumor growth can be efficiently arrested by drugs which stimulate apoptosis [18]. Resveratrol and L-securinine extracted from natural sources have been demonstrated to inhibit tumor growth by initiating apoptotic signals via diverse pathways [19]. This study investigated the anti-proliferative potential of pyrimidine-thioindole on gastric cancer cells. Treatment of AGS and SNU-5 cells with

pyrimidine-thioindole effectively suppressed their proliferation, when compared to control cells. However, pyrimidine-thioindole exposure had no effect on the proliferation of normal GES-1 cells at the two tested concentrations. In AGS and SNU-5 cells, pyrimidine-thioindole exposure significantly increased cellular apoptosis, and enhanced the population of cells in G1 phase, whereas the proportions of cells in S and G2/M phases were reduced. Thus, pyrimidine-thioindole inhibited the proliferation of AGS and SNU-5 cells via generation of apoptotic signals and cell cycle arrest.

The enhancement of expression of miR-145 may serve as therapeutic strategy for cancer because its level is much reduced in different types of cancers [20]. Previous studies have demonstrated that the gastric carcinoma cells i.e. AGS and SNU-5 express higher levels of miR-145 than normal epithelial GES1 cells [15]. The present study showed that in AGS and SNU-5 cells, pyrimidine-thioindole exposure markedly elevated miR-145 expression, with higher elevation in AGS cells than in SNU-5 cells. Carcinogenic properties such as proliferation, progression in cell cycle and metastasis are influenced by the PI3K/AKT pathway [21]. The c-Myc oncogene is involved in transformation of cells, generation of anti-apoptotic signals, and enhanced proliferative potential [22].

Downregulation of the expression of c-Myc induces apoptotic signals, leading to death of cells via damage to DNA [23]. Thus, inhibition of c-Myc expression leads to cell cycle arrest and suppression of cell proliferation. The present study showed that exposure of AGS and SNU-5 cells to pyrimidine-thioindole caused significant downregulations in the expression levels of MMP-2, MMP-9 c-Myc, p-PI3K and p-AKT. The pyrimidine-thioindole-treated AGS and SNU-5 cells expressed higher levels of p21 than untreated cells.

CONCLUSION

These results suggest that pyrimidine-thioindole induces apoptotic signaling, suppresses cell proliferation and arrests cell cycle in AGS and SNU-5 cells. Moreover, exposure to pyrimidine-thioindole in AGS and SNU-5 cells elevates miR-145 expression and targets MMP-2, MMP-9 and c-Myc levels. Therefore, pyrimidine-thioindole can potentially be used for the treatment of gastric cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Rong Zhang and Chunhe Yao performed the experimental work, carried out the literature survey, and analysed and compiled the data. Chunhe Yao designed the study and wrote the manuscript. Both authors read the paper thoroughly and approved it for publication.

Open Access

REFERENCES

1. *Holenz J, Brown DG. Modern lead generation strategies. In Lead Generation: Methods and Strategies: in J. Holenz, R. Mannhold, H. Kubinyi, G. Folkers, Eds, Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany 2016; 13-33.*
2. *Brown DG, Boström J. Where Do Recent Small Molecule Clinical Development Candidates Come From? J. Med. Chem. 2018; 61: 9442-9468.*
3. *Kumar S, Narasimhan B. Therapeutic potential of heterocyclic pyrimidine scaffolds. Chem. Cent. J. 2018; 12: 38.*
4. *Diao PC, Lin WY, Jian XE, Li YH, You WW, Zhao PL. Discovery of novel pyrimidine-based benzothiazole derivatives as potent cyclin-dependent kinase 2 inhibitors with anticancer activity, Eur. J. Med. Chem. 2019; 179: 196-207.*
5. *Farghaly AM, AboulWafa OM, Elshaier YAM, Badawi WA, Haridy HH, Mubarak HAE. Design, synthesis, and antihypertensive activity of new pyrimidine derivatives endowing new pharmacophores, Med. Chem. Res. 2019; 28: 360–379.*

6. Long SA, Thorarensen A, Schnute ME. Pyrimidine and pyridine derivatives useful in therapy. 2013; WO2013054185.
7. Bukowski RM, Yasothan U, Kirkpatrick P, Pazopanib. Nat. Rev. Drug Discov. 2010; 9: 17−18.
8. Hudkins RL, Becknell NC, Zulli AL, Underiner TL, Angeles TS, Aimone LD, Albom MS, Chang H, Miknyoczki SJ, Hunter K. Synthesis and biological profile of the pan-vascular endothelial growth factor receptor/tyrosine kinase with immunoglobulin and epidermal growth factor-like homology domains 2 (VEGF-R/TIE-2) inhibitor 11-(2-methylpropyl)-12,13-dihydro-2-methyl-8-(pyrimidin-2-ylamino)-4H-indazolo[5,4-a]pyrolo[3,4-c]-carbazol-4-one (CEP-11981): a novel oncology therapeutic agent. J Med Chem 2012; 55: 903-913.
9. Ashok P, Lu C-L, Chander S, Zheng Y-T, Murugesan S. Design, synthesis, and biological evaluation of 1-(thiophen-2-yl)-9H-pyrido[3,4-b]indole derivatives as anti-HIV-1 agents. Chem Biol Drug Des 2015; 85: 722-728.
10. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN, 2008. Int J Cancer 2010; 127: 2893-2917.
11. Ghosn M, Tabchi S, Kourie HR, Tehfe M. Metastatic gastric cancer treatment: Second line and beyond. World J Gastroenterol 2016; 22: 3069-3077.
12. Hammond SM. An overview of microRNAs. Adv Drug Deliv Rev 2015; 87: 3-14.
13. Miska EA. How microRNAs control cell division, differentiation and death. Curr Opin Genet Dev 2010; 15: 563-568.
14. Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, Alder H, Liu CG, Oue N, Yasui W, et al. Relation between microRNA expression and progression and prognosis of gastric cancer: A microRNA expression analysis. Lancet Oncol 2010; 11: 136-146.
15. Hu J, Guo H, Li H, Liu Y, Liu J, Chen L, Zhang J, Zhang N. MiR-145 regulates epithelial to mesenchymal transition of breast cancer cells by targeting Oct4. PLoS One 2012; 7: e45965.
16. Lei C, Du F, Sun L, Li T, Li T, Min Y, Nie A, Wang X, Geng L, Lu Y, et al. miR-143 and miR-145 inhibit gastric cancer cell migration and metastasis by suppressing MYO6. Cell Death Dis 2017; 8: e3101.
17. Guimarães RM, Muzi CD. Trend of mortality rates for gastric cancer in Brazil and regions in the period of 30 years (1980-2009). Arq Gastroenterol 2012; 49: 184-188.
18. Lima RT, Busacca S, Almeida GM, Gaudino G, Fennell DA, Vasconcelos MH. MicroRNA regulation of core apoptosis pathways in cancer. Eur J Cancer 2011; 47: 163-174.
19. Fulda S. Modulation of apoptosis by natural products for cancer therapy. Planta Med 2010; 76: 1075-1079.
20. Luo X, Burwinkel B, Tao S, Brenner H. MicroRNA signatures: Novel biomarker for colorectal cancer? Cancer Epidemiol Biomarkers Prev 2011; 20: 1272-1286.
21. Aksamitiene E, Kiyatkin A, Kholodenko BN. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: A fine balance. Biochem Soc Trans 2012; 40: 139-146.
22. Hermeking H, Eick D. Mediation of c-Myc-induced apoptosis by p53. Science 1994; 265: 2091-2093.
23. Maclean KH, Keller UB, Rodriguez-Galindo C, Nilsson JA, Cleveland JL. c-Myc augments gamma irradiation-induced apoptosis by suppressing Bcl-XL. Mol Cell Biol 2003; 23: 7256-7270.