Novel 1,5-diphenyl-6-substituted 1H-pyrazolo[3,4-d]pyrimidin-4(5H)ones induced apoptosis in RKO colon cancer cells

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ABSTRACT

Novel 1,5-diphenyl-6-substituted-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-ones were synthesized and characterized. All compounds were screened for their anti-proliferative activities in five different cancer cell lines. The results showed that compounds 7a and 7b comprising aminoguanidino or guanidino moiety at position 6 inhibited proliferation of RKO colon cancer cells with IC50 of 8 and 4μM, respectively.

Compounds 7a and 7b induced apoptosis in RKO cells, which was confirmed by TUNEL and annexin V-FITC assays. Flow cytometric analysis indicated that compounds 7a and 7b arrested RKO cells in the G1 phase and the most active compound 7b increased levels of p53, p21, Bax, ERK1/2 and reduced levels of Bcl2 and Akt. Compound 7b also activates release of cytochrome c, which is consistent with activation of caspase-9.

Additionally, compound 7b increased caspase-3 activity and cleaved PARP-1 in RKO cells. Collectively, these findings could establish a molecular basis for the development of new anti-cancer agents.

INTRODUCTION

Colorectal cancer (CRC) is the fourth most common form of cancer and the second leading cause for cancer-related deaths1. The prevalence of CRC is still increasing despite enhanced understanding of the pathogenesis of this disease and establishment of improved screening strategies for this malignancy. Current data suggest that epidemiological risk factors for colon cancer, other than genetic risk factors, include smoking, physical inactivity, obesity and food with high fat and carbohydrates that could promote tumor growth2. Thus, the discovery of new therapeutic targets for cancer and development of novel, more effective therapeutic agents remain major challenges in the field of cancer chemotherapy.

The pyrazolo[3,4-d]pyrimidine nucleus is considered as an isostere to the purine nucleus and hence exhibits promising antiproliferative activities. Several members of this family were found to induce apoptosis and/or reduce cell proliferation in many cancer cell lines such as breast, colon, lung and liver cell lines.

To understand the mechanism of action and the effects on cancer biology, we evaluated the effect of the most promising compound on cell cycle, apoptosis and expression of proteins related to cell cycle pathways.

Encouraged by the above-mentioned findings and in continuation of our efforts linked with discovering and exploring novel lead structures as potent chemotherapeutic agents23–26, it was attempted to synthesize new pyrazolo[3,4-d]pyrimidin-4-ones comprising a variety of substituents at position 6 (E; Figure 1) to be evaluated for their anti-cancer activity.

These combinations were suggested in an attempt to investigate the possible synergistic influence of such structure hybridizations on the anticipated biological activity, hoping to discover and develop inducers as potential new anti-cancer agents. apoptosis

MATERIALS AND METHODS

Cancer cell lines used : RKO, colon cancer cells

Drugs: Design and synthesis of l 1,5-diphenyl-6-substituted-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-ones were synthesized and characterized.

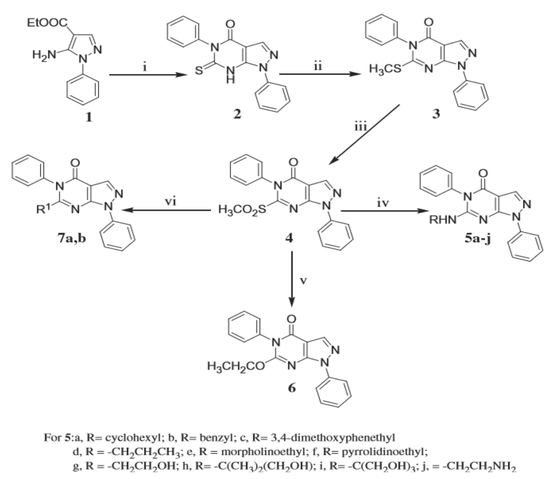
Assays: Cytotoxicity assays; flow cytometry, TUNEL assay, Western blotting, molecular docking experiments

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RESULTS

Synthetic pathways for the target compounds

Scheme 1. Synthetic pathways for the target compounds **5**–**7**.



For **5**:a, R= cyclohexyl; b, R= benzyl; c, R= 3,4-dimethoxyphenethyl
d, R = $-CH_2CH_2CH_3$; e, R = morpholinoethyl; f, R= pyrrolidinoethyl;
g, R = $-CH_2CH_2OH$; h, R= $-C(CH_3)_2(CH_2OH)$; i, R= $-C(CH_2OH)_3$; j, = $-CH_2CH_2NH_2$

Compounds 7a and 7b reduced cell viability and induced apoptosis in RKO cells

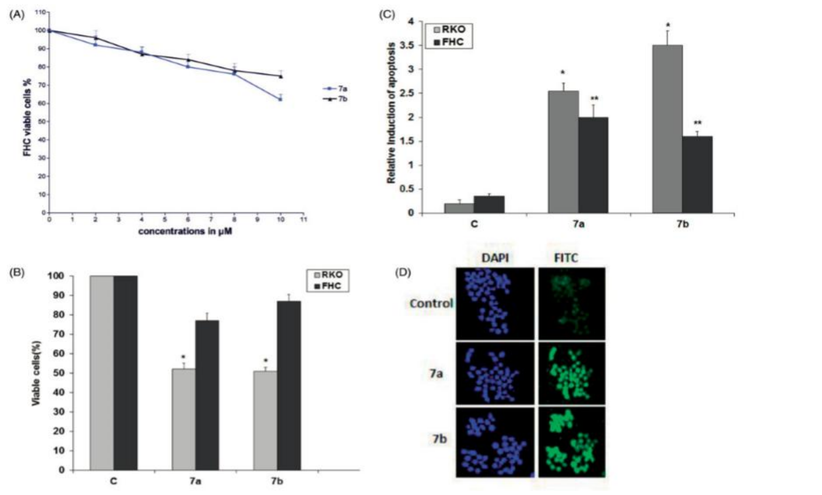


Figure 2. Compounds 7a and 7b reduced cell viability and induced apoptosis in RKO cells. (A) A dose–response curve for compounds 7a and 7b on FHC normal colon cancer cells was constructed. Each data point was an average of results from three independent experiments performed in triplicate and presented as mean±SD. (B) Comparison of 7a and 7b cytotoxicity in FHC and RKO cells. Both cells were treated with 8 and 4μM of compounds 7a and 7b, respectively. Untreated cells were used as control. (C) Comparison of 7a and 7b induced apoptosis in FHC and RKO cells. FHC and RKO cells were treated with 8 and 4μM of compounds 7a and 7b, respectively, for 24h. Untreated cells were used as control. ELISA assay was applied for apoptotic cell detection. Each experiment was performed in triplicate. Data are presented as mean±SD (n¼3). Significant differences between the control and test compounds are indicated by * (*p50.05). (D) TUNEL assay was used to confirm induction of apoptosis in RKO cells. Cells were treated with 8 and 4μM of7a and 7b, respectively, for 24h. Untreated cells act as control. Lack of staining (untreated) cells, represents that the cells are actively proliferating, i.e. without apoptotic cell death and induction of apoptosis in treated cells was confirmed by appearance of TUNEL positive cells. Images were acquired for each fluorescence channel, using suitable filters at 200magnification

Impact of compounds 7a and 7b on cell cycle

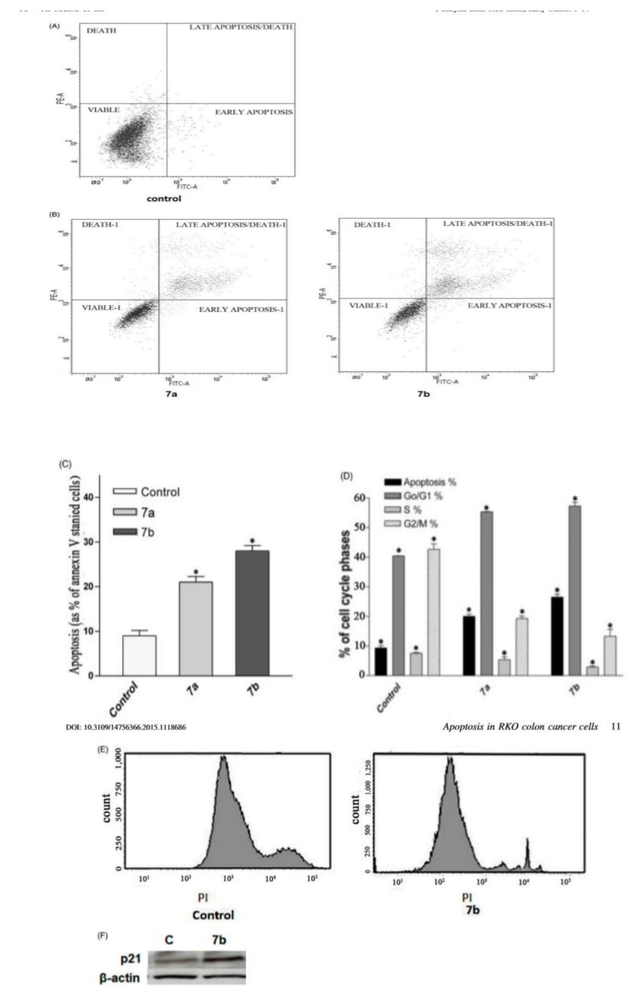
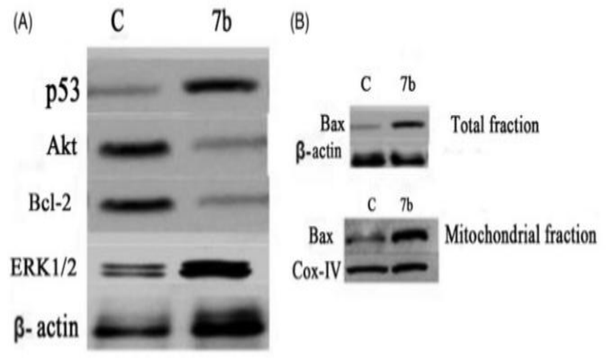


Figure 3. Impact of 7a and 7b on cell cycle. (A and B) Flow cytometric analysis of RKO cells stained with Annexin V-FITC and PI. (A) Untreated RKO cells were used as control, (B) RKO cells treated with 8μM7a and 4μM 7b for 24h. (C) Comparison of 7a and 7b induced apoptosis in RKO colon cancer cells using Annexin V/FITC staining assay. Each bar represents the mean±SD (n¼3). (D) Percent of distribution of cell cycle phases of RKO colon cancer cells after treatment with 7b for 24h. (E) A representative image of flow cytometric analysis showing G1 arrest. (F) Total protein was extracted from RKO cells and used for Western blotting analysis to determine the differences in p21 between untreated cells (control; C) and cells treated with 7b for 24h. b-Actin was used as loading control.

RESULTS AND CONCLUSION

Effect of compound 7b on the expression level of signaling proteins



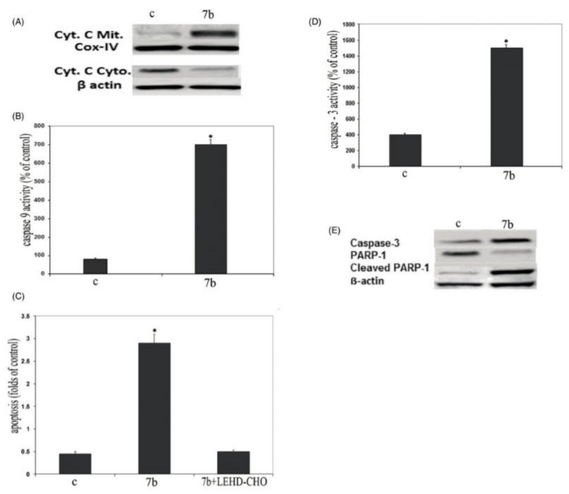


Figure 4. Compound 7b induced apoptosis through initiation of the mitochondrial pathway. (A) Western blot analysis was used to determine the release of cytochrome c in the cytosol and mitochondria of 7b-treated RKO cells. b-Actin was used as loading control and IV-Cox was used as internal mitochondrial control. C: control (untreated cells). (B) Effect of 7b on the level of caspase-9 in RKO cells after treatment with 4μM 7b for 24h. (C) Effect of 7b and its combination with caspase-9 inhibitor on 7b-induced apoptosis. Cells were preincubated with 20mM LEHD-CHO for 1 h before the addition of 4μM of7b for an additional 24 h. Induction of apoptosis was determined at 405nm and compared to control (untreated cells). (D) Activation of caspase-3 in RKO cells after treatment with 4μM 7b for 24h. (For B, C and D; each data point is the mean of three independent experiments and expressed as M±SD. The asterisk indicates a significant difference between control (C; untreated cells) and 7b-treated cells. (E) Western blot analysis was used to determine the expression levels of caspase-3 and cleaved PARP1 after treating cells with 7b for 24h. b-Actin was used as loading control.

SUMMARY

In the present investigation, new 1,5-diphenyl-6-substituted 1Hpyrazolo[3,4-d]pyrimidine-4(5H)-ones were synthesized and screened for their anti-tumor activity in five cancer cell lines. Results revealed that compounds 7a and 7b were the most active derivatives in this study against RKO colon cancer cells and they showed less cytotoxic effect on normal colon cells (FHC) than colon cancer cells (RKO).

The results of apoptosis assays and cell cycle analysis demonstrated that compounds 7a and 7b obviously inhibit the proliferation of RKO cancer cells by inducing apoptosis and arresting the cell cycle at G1 phase. The most active compound 7b increased levels of p53, p21, Bax, ERK1/2 and decreased the level of Bcl-2 and AKT. The regulatory effects of derivative 7b on the Bcl-2 family are correlated with the release of cytochrome c from the mitochondria into the cytoplasm and the activation of caspase-9. Compound 7b also increased caspase-3 and cleaved PARP-1 in RKO colon cancer cells.

Finally, it could be concluded that compound 7b was found to induce apoptosis in RKO colon cancer cells via modulating mitochondrial signaling pathway. Accordingly, compound 7b was identified as a new lead compound that merits further optimization and development as an anti-cancer candidate.

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