EUR. J. ONCOL.; Vol. 20, n. 1, pp. 17-24, 2015

Inhibition of growth and induction of differentiation and apoptosis in human leukemia K562 cells by a new compound from dihydropyrano[c]chromenes family

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Summary. *Aim:* It has been reported that derivatives from the chromene family have potent anti-leukemic activity. Differentiation therapy is a promising treatment for myeloid leukemia. Herein, we evaluated inhibition of growth, differentiation induction and apoptosis in human myeloid leukemia K562 cells by the novel derivatives of dihydropyrano[c]chromenes. *Methods:* K562 cells were treated with different concentrations of the new dihydropyrano[c]chromenes (20-260 μM) derivatives for 3 days. To investigate growth inhibition and viability of the cells, a trypan blue exclusion assay was applied. Differentiation was investigated morphologically by wright-Giemsa staining and latex particle phagocytosis assay. Apoptosis was observed by morphological criteria, the acridine orange/ethidium bromide (AO/EtBr) double staining method, as well as DNA ladder formation. *Results:* The IC_{50} values of the 4-PC, 4-NC, 4-CNC and 4-HC after 48 h of exposure was 240±4.5, 60±3.5, 180±4.2 and 160±5.5 μM for K562 cells, respectively. 4-NC was found to be the most effective compound and was chosen for further studies. 4-NC inhibited growth and proliferation in a dose- and time-dependent manner. Moreover, our evidence showed that the 4-NC effects on K562 cells resulted in differentiation toward a monocyte/macrophage lineage. The data from the AO/EtBr and DNA fragmentation assay confirmed qualitatively that K562 cell treatment with 4-NC induces apoptosis. *Conclusion:* Based on our current observations, these compounds can be valuable candidates for effective chemotherapy acting through differentiation induction and apoptosis.

Key words: apoptosis, differentiation, dihydropyrano[c]chromenes, leukemia, K562 cells

«Inibizione della crescita, induzione del differenziamento ed apoptosi nelle cellule umane leucemiche tipo K562 tramite un nuovo composto derivato dalla famiglia *dyhidropyrano[c]chromenes*»

Riassunto. *Scopo:* In letteratura è riportato che i derivati della famiglia dei *cromenes* hanno una potente attività anti-leucemica. La terapia differenziata è un trattamento promettente per la leucemia mieloide. Qui di seguito abbiamo valutato l'inibizione della crescita, l'induzione del differenziamento e l'apoptosi nelle cellule umane di leucemia mieloide tipo K562 tramite nuovi composti derivati da *dyhidropyrano[c]chromenes*. *Metodi:* Le cellule tipo K562 sono state trattate con differenti concentrazioni dei nuovi derivati da *dyhidropyrano[c] chromenes* (20-260 μM) per tre giorni. Per studiare l'inibizione della crescita e la vitalità delle cellule, è stato eseguito un test di esclusione del *trypan blue.* Il differenziamento è stato studiato morfologicamente tramite colorazione *wright-Giemsa* e test di fagocitosi di particelle di lattice. L'apoptosi è stata studiata tramite criteri morfologici, metodo a doppia colorazione, *acridine orange/ethidium bromide* (AO/EtBr), così come per la

formazione della struttura del DNA. *Risultati:* I valori di IC_{50} di 4-PC, 4-NC, 4-CNC e 4-HC dopo 48 ore di esposizione erano 240±4.5, 60±3.5, 180±4.2 e 160±5.5 μM per le cellule K562, rispettivamente. 4-NC è risultato essere il composto più efficace ed è stato scelto per ulteriori studi. 4-NC ha inibito la crescita e la proliferazione in maniera dose e tempo dipendente. Inoltre, i nostri risultati hanno dimostrato che gli effetti di 4-NC sulle cellule K562 hanno portato al differenziamento verso la linea monocita/macrofago. I dati provenienti dai test AO/EtBr e dalla frammentazione del DNA hanno confermato qualitativamente che il trattamento delle cellule K562 con 4-NC induce apoptosi. *Conclusioni: S*ulla base delle nostre attuali osservazioni, questi composti possono considerarsi validi candidati per una chemioterapia efficace che agisce attraverso l'induzione del differenziamento e dell'apoptosi.

Parole chiave: apoptosi, differenziamento, *dyhidropyrano[c]chromenes,* leucemia, cellule K562

Introduction

Induction of differentiation as a therapeutic approach is considered to have powerful effects on Chronic Myelogenous Leukemia (CML) (1). The human CML K562 cell line serves as an *in vitro* model for hematopoietic differentiation (2). Differentiation therapy represents a valuable treatment for CML which can be induced by several differentiation agents including hemin (3), 5-azacytidin (4), l-β-D-arabinofuranosylcytosine (5), daunomycin (6), or herbimycin A (7) which are considered to induce erythroid differentiation in K562 cells. In contrast, treatment of K562 cells with phorbol esters (phorbol 12-myristate 13-acetate) can induce differentiation along a megakaryocytic-monocytic lineage (8, 9). Apart from differentiation, various compounds cause apoptosis among treated leukemia cells (10-13). Apoptosis can occur as part of the normal physiological process or in the pathological deletion of cells to regulate the balance between cell proliferation and cell death (14). During apoptosis, various characteristic changes occur within the cells, including condensation of the nucleoplasm and cytoplasm, chromosomal DNA fragmentation, and the formation of membrane-bound apoptotic bodies, which are rapidly recognized and eliminated by adjacent cells (15). It is thought that differentiation-induced apoptosis in proliferative tumor cells may contribute to cancer chemotherapies (10). Since many cancerous cells exhibit abnormal inhibition of apoptosis, researchers are interested in the discovery and development of apoptotic inducers which serve as potential anti-cancer agents (16). Dihydropyrano[*c*]chromenes and their derivatives have been found to possess many interesting biological properties including anti-cancer, anti-Alzheimer's disease, and anti-HIV activities. Additionally, they have proved effective for the treatment of AIDS associated dementia, Down's syndrome and schizophrenia (17-20). To further identify the potential anti-cancer properties of structurally different derivatives of the dihydropyrano[c]chromene family, we decided to investigate the effect of four new compounds from these derivatives on growth rate and viability of K562 cells. Moreover, induction of differentiation and apoptosis was evaluated in these cells by an active compound from this family.

Materials and methods

Materials

The cell culture medium (RPMI-1640), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL Life Technologies (Paisley, Scotland). The culture plates were obtained from Nunc (Roskilde, Denmark). Wright-Giemsa stains were purchased from Sigma Chemical (St Louis, MO, USA). Ethidium bromide and RNase were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). The cell line (K562) was obtained from the Pasteur Institute of Iran (Tehran).

General procedure for preparation of dihydropyrano[c] chromene compounds

Four dihydropyrano[c]chromene compounds including 2-amino-5-oxo-4-phenyl-4H, 5H-pyrano-[3,2-c]chromene-3-carbonitrile (4-PC), 2-amino-4-(4-nitrophenyl)-5-oxo-4H, 5H-pyrano-[3,2-c]chromene-3-carbonitrile (4-NC), 2-amino-4-(4-cyano phenyl)-5-oxo-4H, 5H-pyrano-[3,2-c]chromene-3-carbonitrile (4-CNC) and 2-amino-4-(4-hydroxyphenyl)-5-oxo-4H, and 5H-pyrano-[3,2-c]chromene-3-carbonitrile (4-HC) were prepared, according to the previously described method (21).

Cell culture

Erythroid leukemia K562 cells were routinely cultured in RPMI 1640 medium containing 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. Cultured cells were kept in a humidified atmosphere containing 5% CO2 at 37˚C (22).

Viability and growth inhibition

The cytotoxicity and growth inhibition of the Dihydropyrano[c]chromene family was determined by routine trypan blue dye. Briefly 1×10^5 cells were seeded in 96-well plates with 200 μl of the medium. After 24 h, they were treated with different concentrations of the drug for various times. For each concentration, the cytotoxicity and growth inhibition were measured in triplicate and the average value was taken (23).

Morphological study of the apoptotic cells

Morphological change in apoptotic cells was studied using the fluorescent dyes Acridine Orange and Ethidium Bromide (AO/EtBr). These dyes intercalate with DNA and are used to identify nuclear morphology of K562 cells. The K562 cells (1×10^5 cells/well) were cultured in 24-well plates and treated with the Dihydropyrano[c]chromene family at the concentrations indicated (IC_{50} Values) for 72 h. Following treatment, the cells were collected and washed with cold PBS. Then 20 μl of staining solution (1:1) mixture containing 100 μg/ml of EtBr and 100 μg/ml of AO was added to the cell suspension in a final concentration of 100 μg/ml. The morphological change was then evaluated by ultraviolet fluorescence microscopy (Zeiss, Germany) (11).

DNA fragmentation assay

For detection of DNA fragmentation, K562 cells were treated with a concentration (IC_{50} Values) of the active compound 4-NC as indicated for 48h. Briefly, 2×10^6 cells were cultured and after drug treatment, total DNA was extracted from treated and untreated cells using the Proteinase K/RNase A extraction method; then the cells collected were washed twice with cold PBS, adding 100 μl lytic buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) as indicated by the manufacturer. After centrifugation, the samples were resuspended in 10 μl of 10 mg/ml RNase A for 1 h at 37˚C followed by suspension in 10 μl of 20 mg/ml proteinase K for 2 h at 50 ˚C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and then washed in 70% ethanol. Finally DNA fragmentation was visualized by electrophoresis in a 2% agarose gel via staining with ethidium bromide, and exposed to UV light (24).

Morphological study of the differentiated cells

Morphological differentiation of cells can be characterized by Wright–Giemsa staining. K562 cells were cultured at a density of 1×10^5 cells/ml in a 12-well plate and incubated in RPMI 1640 with 10% FBS. After treating cells with single doses (at IC_{50} Values) for 24 h, treated and untreated cells were harvested and fixed with methanol, then stained with Wright-Giemsa. The morphological changes in control and treated cells were examined under a light microscope at high magnification (400×).

Latex particle phagocytosis assay

Phagocytic leukocytes including monocytes and macrophages constitute a first line of host defense via phagocytosis of microorganisms (25). The phagocytosis ability was detected using protein-coated latex particles. To assay the ability of the cells to phagocyt-

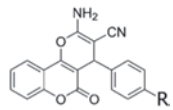
ize protein-coated latex particles, a particle suspension (Gravindex-Ortho, Omega House, UK) was diluted 1:10 with PBS. Then, the treated and untreated cells (5×10^4 cells), suspended in a medium containing 0.1 ml of RPMI 1640 supplemented with 20% FBS, were mixed with 0.1 ml of the diluted suspension. The resulting mixture was incubated for 1h in the CO_2 incubator. After incubation, the cells were washed three times with cold PBS and resuspended in PBS. A minimum of 200 cells were counted in triplicate and those with a minimum of 10 digested particles were considered positive (25).

Results

Cell viability and growth inhibition in K562 cells

The K562 cells were treated with different doses of the drug for various time intervals up to 72 h and their growth patterns were established by trypan blue exclusion test. As shown in Fig. 1, the dihydropyrano[c] chromene derivatives inhibited the growth of K562 cells in a dose- and time-dependent manner. The IC_{50} values of 4-PC, 4-NC, 4-CNC and 4-HC after 48 h of exposure were 240±4.5, 60±3.5, 180±4.2 and 160±5.5 μM for K562 cells, respectively (Table 1). Despite the growth inhibiting activity of the dihydropyrano[c] chromene derivatives at 60-240 μM, no significant changes in the viability of the treated cells were observed after 24 h of treatment; however, after 48 h of incubation the viability of the treated cells decreased until massive cell death after 72h (Fig. 1).

Table 1. Growth inhibition activity (IC_{50}, μM) of dihydropyrano[c]chromene derivatives against K562 cell lines. [a]Data are means of three or more experiments and are reported as means ± standard error of the mean (SEM).



Compound	R	IC_{50} [a] (μM)
4-PC	H	240±4.5
4-NC	NO_2	60±3.5
4-CNC	CN	180±4.2
4-HC	OH	160±5.5

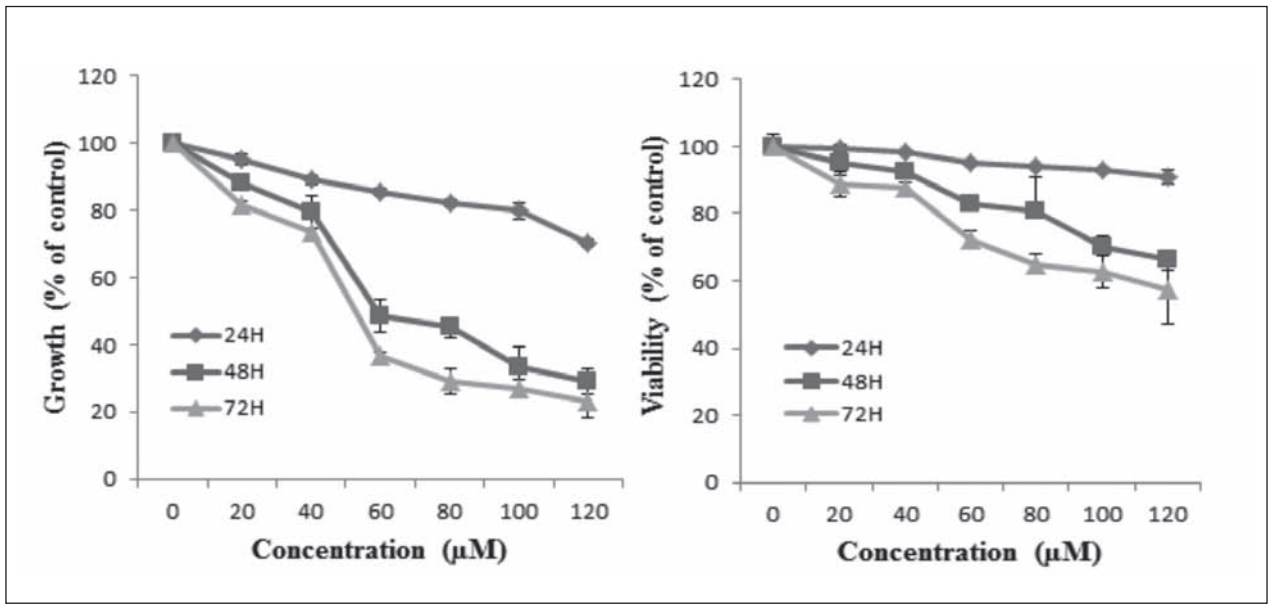


Figure 1. Effects of 4-NC on growth and viability of K562 cells. Cells were exposed to the indicated amounts of 4-NC (20-120 μM) for 24, 48 and 72 h (results of other compounds not shown). The viable cells were also evaluated by trypan blue exclusion at various time intervals. Cell viability and growth inhibition in each treatment were expressed as a percentage of the controls. Each value represents the mean ± SD of three independent experiments ($P<0.05$).

Induction of differentiation in K562 cells

Phase contrast microscopic examination of the drug-treated K562 cells revealed distinct alterations in cell morphology. The effects of dihydropyrano[c] chromene derivatives on the morphology of K562 cells in the course of time were studied 24 h after drug exposure, a portion of the cells forming aggregates, while the majority of the cells developed pseudopodia extensions attached to the culture dishes (Fig. 2). These morphological changes seem very similar to monocyte/macrophage differentiation of leukemia cell lines (26). In addition, the morphologic variations and phagocytic activity were analyzed using the Wright-Giemsa staining method. Fig. 2 shows the morphologic features of the macrophage lineage, such as significant changes in cell size and an increase in cytoplasm to nuclear ratio (Fig. 3). After 48 h treatment with 4-NC, a large population of the treated cells showed macrophage features (Fig. 3). We also evaluated the phagocytic activity of the cells affected. It is well established that mature myeloid cells are generally more active in phagocytosis than their corresponding progenitor cells (27). Phagocytosis of latex particles, which is commonly considered as a criterion of proper function of mature macrophages, indicated that treated cells were able to phagocytize latex particles after 48 h treatment with a single dose (60 μM) of 4-NC compound (Fig. 4).

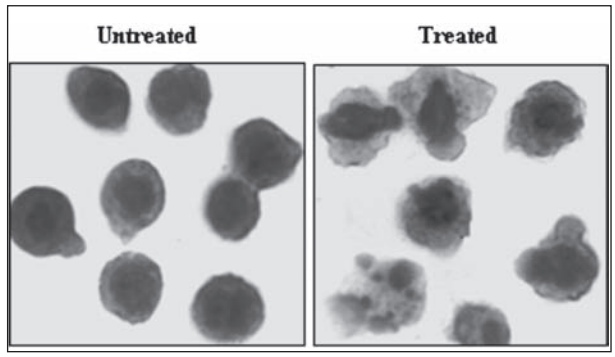


Figure 3. Morphological changes of erythroleukemia K562 cells treated with 4-NC. After 48 h treatment of K562 cells with 60 μM of 4-NC, the cells were collected, stained by Wright-Giemsa solution and studied using a light microscope.

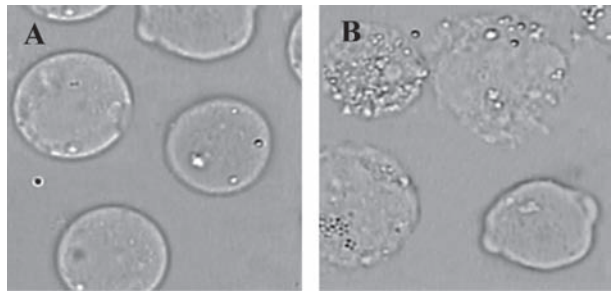


Figure 4. Phagocytic activity in chronic promyelocytic K562 cells. Phagocytic activity of the differentiated cells was studied after 48 h of treatment. (A) Control cells treated after 72 h. (B). Treated cells incubated with protein-coated latex particles, as described in "Materials and Methods". Magnification, 400×

Induction of apoptosis in K562 cells

As stated previously, treatment of the K562 cells with the dihydropyrano[c]chromene derivatives decreased the viability of the cells. To verify whether cell death by the new compounds was the result of apoptosis, we assessed the nuclear morphological changes by the fluorescence technique after double staining with AO/EtBr. As shown in Fig. 5A, K562 cells un-

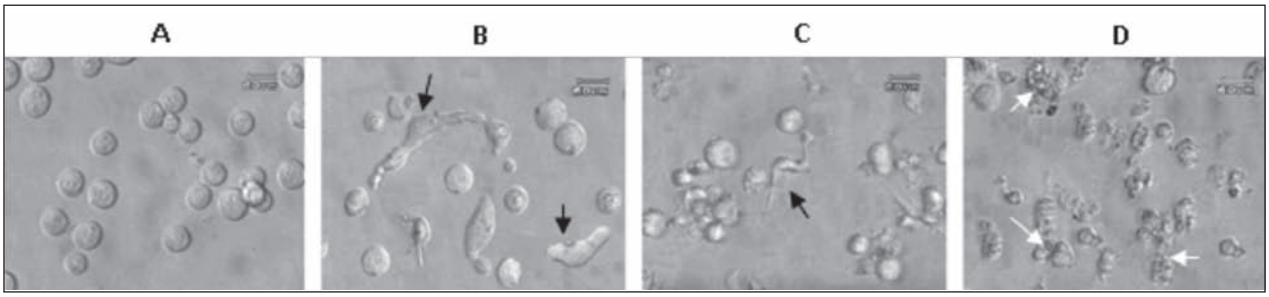


Figure 2. Morphological changes in K562 cells treated with 4-NC (60 μM) for 24-72 h. Control cells (A), treated cells after 24 (B), 48 (C) and 72 (D) h of exposure to the drug. Formation of pseudopodia among differentiated cells and the occurrence of cell death are shown with black and white arrows, respectively. Magnification, 100×.

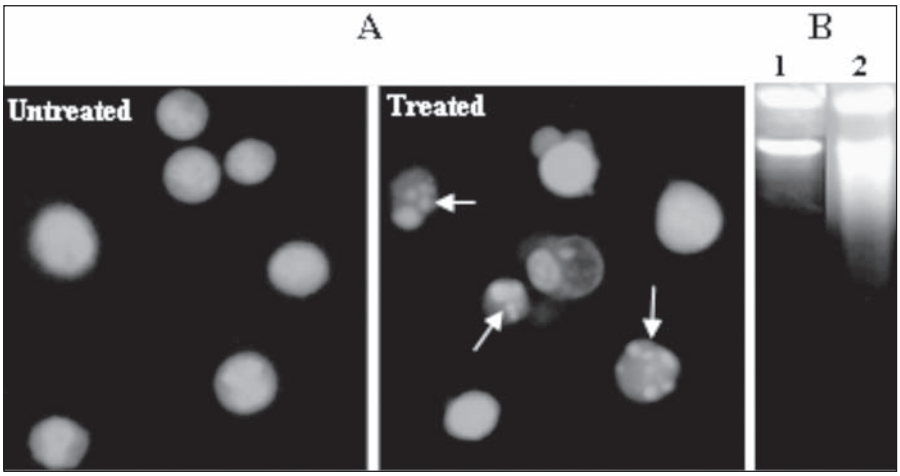


Figure 5. Induction of apoptosis in K562 cells by 4-NC. (A) The drug treated Cells (60 μM, after 72 h) were stained with acridine orange/ethidium bromide and then observed by fluorescent microscopy (200×). The compound induced chromatin condensation and nuclei fragmentation (arrows). (B) DNA fragmentation after 72 h treatment of K562 cells with 60 μM of 4-NC. Lane 1: untreated cells; Lane 2: treated cells.

derwent apoptosis upon a single dose (at IC_{50} value) of 4-NC after 72 h. The occurrence of apoptosis was further documented by observing a laddering pattern in the gel electrophoresis of DNA obtained from drug-treated K562 cells (Fig. 5B). These data indicated that derivatives of the dihydropyrano[c]chromene family cause apoptosis after induction of differentiation in K562 cells.

Discussion

Chronic myelogenous leukemia (CML) is a clonal disorder of hematopoiesis that arises in a hematopoietic stem cell or early progenitor cell. This is characterized by the dysregulated production of mature non-lymphoid cells with normal differentiation (28). Thus, a possible therapeutic strategy for treatment of leukemia would be to induce terminal differentiation and eventual senescence. It has been reported that derivatives from the chromene family have potent anti-leukemic activity *in vitro* and *in vivo* (29). Our results in Fig. 1 suggested that dihydropyrano[c]chromene derivatives have growth inhibitory effects. More specifically, the IC_{50} values of 4-NC, 4-CNC and 4-HC were lower than the IC_{50} value of the reference compound (4-PC) in K562 cells. The presence of electron-withdrawing groups of OH, CN and NO_2 in the C-4 position of the phenyl ring appeared to confer high growth inhibitory activity as compared with the reference compound which had an H group in the C-4 position of phenyl ring. The results of growth inhibition shown that these compounds have weak-to-moderate *in vitro* anti-cancer effects.

K562 cells can be regarded as pluripotent hematopoietic progenitor cells expressing markers for erythroid, granulocytic, monocytic, or megakaryocytic lineages (30). The current morphological and Latex particle phagocytosis experiments indicated that the new compounds at IC_{50} values in K562 cells significantly induced differentiation along the monocytic/macrophage lineage. Our observations indicated that, after approximately 12 h, the cells began to differentiate and the peak was reached after 2 days of drug exposure. It has been reported that apoptosis plays an important role in the elimination of activated granulocytes and monocytes (31, 32). This is because the mature blood cells have a short life span both *in vitro* and *in vivo*, and hence die as a result of apoptosis (32). The results obtained from AO/EtBr staining and the DNA fragmentation assay confirmed the occurrence of apoptosis after cell differentiation. Apoptosis, like differentiation, was also dose- and time-dependent. Apoptosis began after 24 h at high drug dose exposure and increased to its highest level after 72 h.

Conclusion

In conclusion, the results of this investigation clearly indicate that the new and active compound of dihydropyrano[c]chromene is capable of inducing differentiation and apoptosis in K562 cells and therefore may be regarded as a valuable candidate for pharmaceutical evaluation.

Acknowledgement

The authors appreciated financial support by the research council of Tabriz University

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Received: 28.10.2014
Accepted: 5.3.2015
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