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Original Article

The K562 Chronic Myeloid Leukaemia Cell Line undergoes Apoptosis in Response to 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine

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Abstract

Background & Aims: Leukemia is a particular type of cancer characterized by the failure of cell death or disability in the differentiation of hematopoietic cells. Chronic myelogenous leukemia (CML) is the most studied kind of this type of cancer. In this study, the anti-cancer effect of the 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine compound on the human leukemia K562 cells was investigated.

Materials & Methods: The K562 cell line was cultured by initially seeding 1×10^6 cells per milliliter in RPMI 1640 medium. Cell viability was investigated using trypan blue exclusion and MTT assays. Cell death in cancer and normal cells was quantified using propidium iodide (PI) and acridine orange (AO) double staining. The one-way analysis of variance (ANOVA) and the SPSS16 and Excel softwares were used for data analysis. Data were analyzed statistically using the SPSS16 software. A probability level of $p<0.05$ was considered as the statistically significant reference.

Result*:* The 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine compound had a strong fatal and concentration-dependent effect on K562 cells and caused cell death mainly through induction of apoptosis. Statistical analysis of the cells under a fluorescence microscope revealed significant differences in apoptotic cell populations between treated and untreated cells.

Conclusions*:* The results of this investigation indicated that the 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine compound does have cytotoxic effects on the K562 cell line. This information also revealed that this compound may initiate a new therapeutic standpoint for the treatment of leukemia.

Keywords**:** 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine, apoptosis, K562 tumor

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Introduction

The search for new anticancer agents continues to be an active area of research at many research centers (1). Previous researches had shown that pyrimidine compounds can prevent many cancers (2). The results of the use of 2-4 dichloropyrimidine showed that this derivative could be potentially useful in combating K562 human cell line cancer without cytotoxic effects on peripheral blood mononuclear (PBMC) cells (3). Chronic myeloid leukemia (CML) is one of the most well-known cancers of the blood that occurs in the stem cells due to a bilateral mutation between the abl gene and the bcr gene on chromosome 22. The K562 cell line is used as a study pattern (CML) (4). Apoptosis is a

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regulated process of cell death that removes unwanted cells without damaging and devastating impacts on the cells (5). All treatments, including surgery, amputation, radiotherapy, and chemotherapy, have many side effects, including hair loss, nausea, vomiting, itchy skin, physical weakness, and an increased risk of infection following a weakened immune system (6). The aim of this study was to evaluate whether 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine would be an option for treatment of the patients with CML, and for this, we incubated the K562 cell line with this compound.

Material & Method

In this study, K562 tumoral cell line (Pasteur Institute, Iran) and peripheral blood mononuclear cells (PBMCs) in the hematology laboratory cell bank were used. After recovery, frozen cells (tumoral and normal PBMCS cells) were prepared for testing under sterile conditions. Also, PBMCs from a healthy individual were collected and used as the control (normal cells). Cells were cultured by initially seeding 1×10^6 cells per milliliter in RPMI 1640 medium (GIBCO-BRL) containing 10% heat-inactivated fetal bovine serum (GIBCO-BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin (Bioidea) in a humidified incubator at 37°C with 5% CO_2 (7). The number of viable cells was determined by trypan blue dye exclusion test using a hemocytometer. Cells with viability greater than 95% were used for the experiment (8). MTT assay test was performed to determine the effect of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine derivative in K562 cells and an IC50 value was obtained. Briefly, appropriate numbers of cells (10,000 cells) were cultured in 96-well plates. After 24 hours of incubation, different doses (serial dilution) of the compound (1.5625-200 µg/ml) were added to each well (9). At least three wells were allocated for each concentration. After 48 hours, an MTT test was performed. When the MTT solution was absorbed by the living cells, a formazan product with an absorbance maximum at 498 nm was produced; optical density (OD) will show the number of the cells per well. The OD of formazan was measured using spectrophotometry (10). The IC50 value was calculated as the sample concentration that caused 50% cell death as compared to the control cells (no drug added).

Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining:

Cell death in cancer and normal cells was quantified using propidium iodide (PI) and acridine-orange (AO) double staining, according to the standard procedures, and examined under a fluorescence microscope (10). Briefly, treatment was carried out in a 25 mL culture flask. K562 cells were plated at a concentration of 1×10^6 cell/mL and treated with this pyrimidine derivative at IC50 concentration. Flasks were incubated in an atmosphere of 5% CO_2 at 37°C for 24, 48, and 72 hours. The cells were then spun down at 400 rpm for 10 minutes. The supernatant was discarded and the cells were washed twice using phosphate buffer saline (PBS) after centrifuging at 400 rpm for 10 minutes to remove the remaining media (11). 10 microliters of fluorescent dyes containing acridine orange (AO; 10 µg/mL) and propidium iodide (PI; 10 µg/mL) were added to the cellular pellet at equal volumes each. The freshly stained cell suspension was dropped into a glass slide and covered by a coverslip. Slides were observed under a UV-fluorescence microscope within 30 minutes before the fluorescence color starts to fade. The percentages of viable, apoptotic, and necrotic cells were determined in more than 200 cells. Acridine orange and propidium iodide are intercalating nucleic acid-specific fluorochromes which emit green and orange fluorescence, respectively, when they are bound to DNA. Viewed by fluorescence microscopy, viable cells appear to have a green nucleus with intact structure, while apoptotic cells exhibit a bright-green nucleus showing condensation of chromatin as dense green areas. Late apoptotic cells and necrotic cells will stain with both AO and PI. Comparatively, PI produces the highest intensity emission. Hence, late apoptotic cells exhibited an orange nucleus showing condensation of chromatin whilst necrotic cells display an orange

nucleus with intact structure (11). This assay provides a useful quantitative evaluation and was done three times (n = 3).

Statistical analysis:

Data were analyzed statistically using the SPSS16 software. The one-way analysis of variance (ANOVA) is used to determine whether there are any significant differences between the means. A probability level of $p<0.05$ was considered as the statistically significant reference.

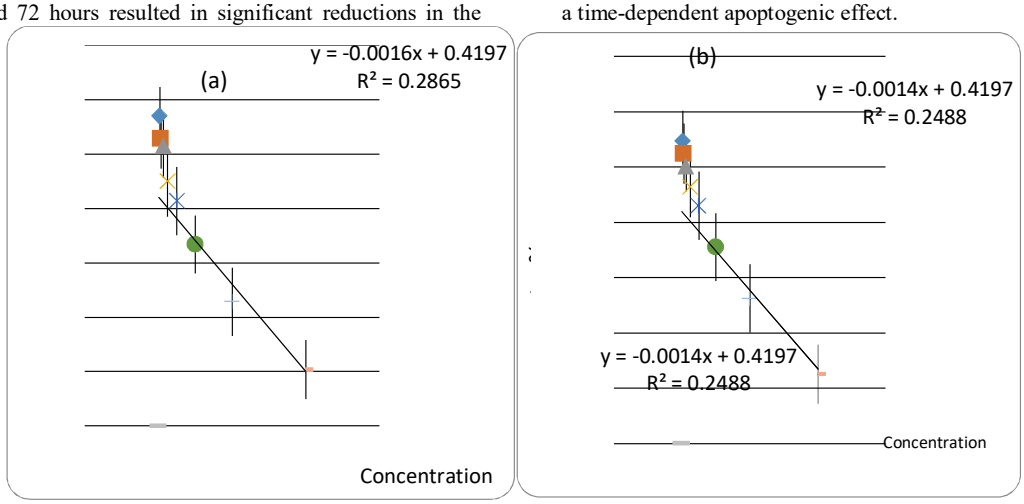
Results

Effects of 4-Chloro-5-Iodo-7H-Pyrrolo[2.3-D]Pyrimidine Derivatives on Cell Viability:

The K562 and PBMC cells were incubated with various concentrations of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine for 24, 48, and 72 h. The impact of the novel pyrimidine compound on cell viability was quantitated 48 h after exposure by the MTT assay. Exposure of K562 cells to 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine significantly inhibited their growth in a dose- and time-dependent manner ($p<0.05$). While there was no significant effect from the low concentration of the novel pyrimidine compound after 24 hours, there were significant decreases in viability at concentrations (serial dilution) of (1.5625-200 µg/ml). Treatment of tested pyrimidine derivative cells for 48 and 72 hours resulted in significant reductions in the number of viable cells (Figure. 1 and 2). The IC50 dose for the 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine cells was determined to be 1.45±0.125 µg/ml, 1.57±0.132 µg/ml, and 1.74±0.111 at 24, 48 and 72 h, respectively (Table1). The IC50 value of K562 cells was lower compared to the PBMC cells. So, the novel pyrimidine compound had greater cytotoxicity against cancer cells.

Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining:

Apoptotic, necrotic, and live K562 cells were scored under the fluorescence microscope. This also included the control (untreated) cells whereby, 200 cells were randomly and differentially counted. The study revealed that 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine triggered morphological features that was related to the apoptosis in a time-dependent manner (Figure. 3 and 4). Whereby, early apoptosis is obvious by intercalating AO within the fragmented DNA. In contrast, untreated cells were observed with a green intact nuclear structure. During 48-hour treatment with the tested pyrimidine derivative, blebbing and nuclear margination were noticed (moderate apoptosis). Differential scoring of treated 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine cells (200 cells population) showed that there was a statistically significant difference in apoptosis-positive cells ($P< .001$), which indicates clearly that it does have a time-dependent apoptogenic effect.



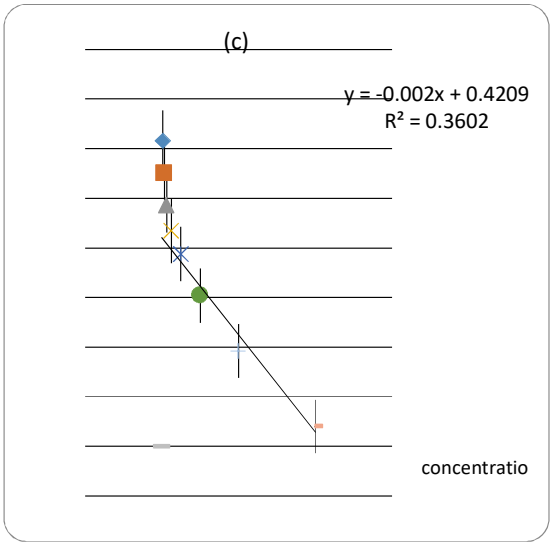
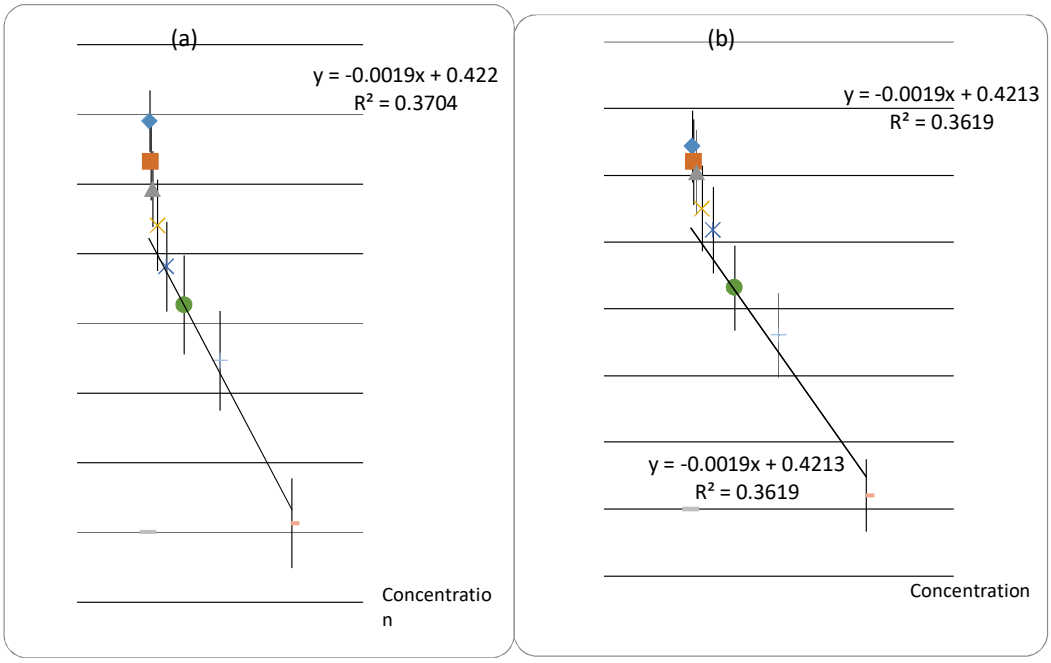


Fig 1. Effect of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine on cell viability of K562 cells. Cells were treated with different concentrations of the tested derivative for 24 (a), 48 (b), and 72 (c) hours. Viability was quantitated by MTT assay. Results are shown as mean±SEM.



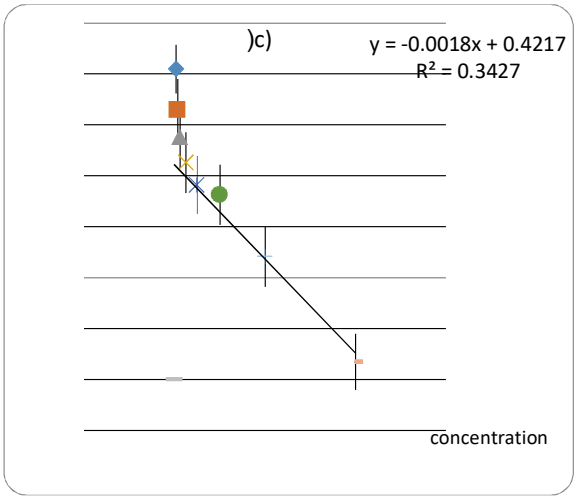


Fig 2. Effect of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine on cell viability of PBMC cells. Cells were treated with different concentrations of tested pyrimidine compound for 24(a), 48 (b), and 72(c) hours. Viability was quantitated by MTT assay. Results are shown as mean±SEM.

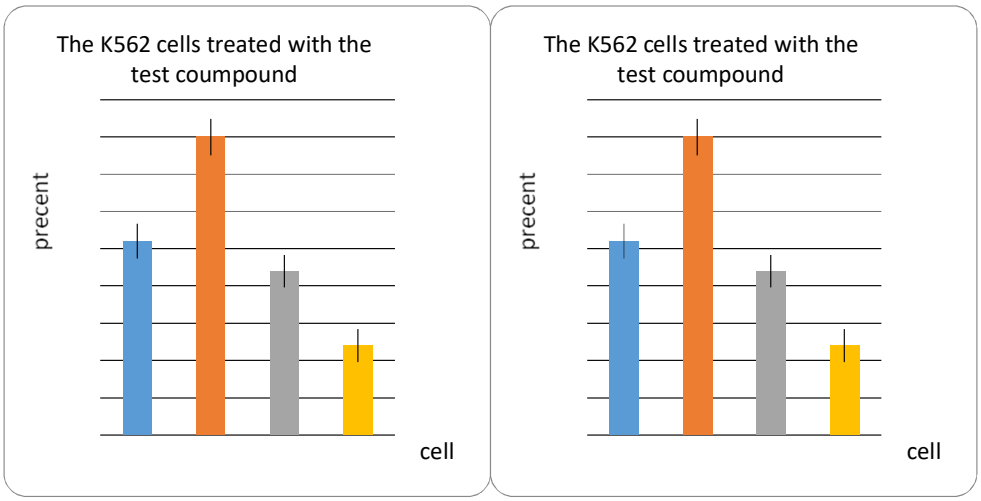


Fig 3: Assessment of apoptosis by propidium iodide (PI) and acridine orange (AO) double staining.

In the present study, exposure of K562 cells to 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine significantly inhibited their growth in a dose- and time-dependent manner ($p<0.05$). For this novel pyrimidine compound, significant inhibitory effects on proliferation were only observed for the highest concentration (200 µg/ml) after 24 h exposure. However, there were significant decreases in viability after 48 and 72 h. In addition, our results revealed that the studied pyrimidine derivative triggered morphological features that relate to apoptosis in a time-dependent manner. The 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidines had greater cytotoxicity against the cancer cells compared to the normal cells. These results are in line with the other reports about similar compounds.

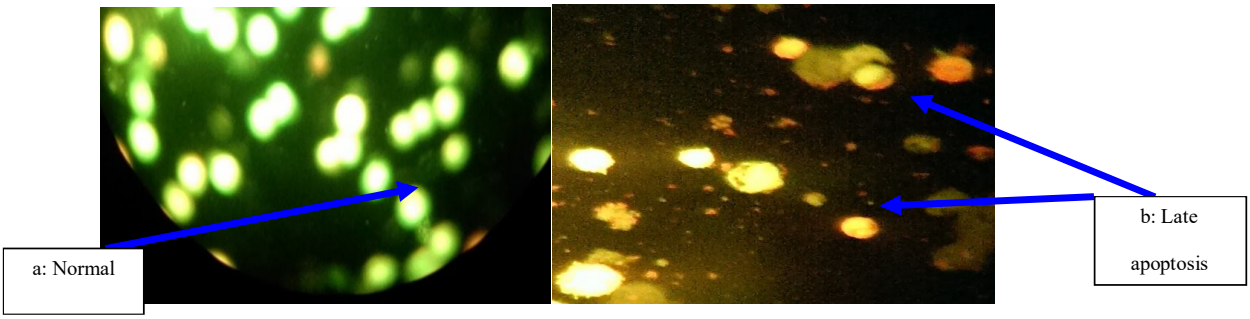


Fig 4. Fluorescent micrograph of acridine-orange and propidium iodide double-stained K562. The K562 cells were treated at IC50 of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine in a time-dependent manner. Cells were cultured in RPMI 1640 media (25 mL flask), maintained at 37°C and 5% CO_2. (a) Untreated cells showed normal structure without prominent apoptosis and necrosis. (b) Late apoptosis was seen in 48 hours incubated cells, whereby positive staining with orange color represents the hallmark of late apoptosis (magnification400X).

Table 1. Doses of 44-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine and busulfan as the control drug as well inhibition of the cell growth by 50% (IC50) in the leukemia cancer cell line K562, PBMC

	24h		48h		72h	
	IC50 (µg/ml) ±S.D	SI	IC50 (µg/ml) ±S.D	SI	IC50 (µg/ml) ±S.D	SI
K562	1.45 ±0.125	1.336	1.57 ±0.132	1.356	1.74 ±0.111	1.183
PBMC	2.31 ±0.092		2.04 ±0.086		2.41 ±0.078	
busulfan						
K562	1.10 ±0.052	2.977	0.869 ±0.061	2.807	0.84 ±0.053	2.726
PBMC	3.275		2.44		2.29	

By dividing the numerical value of IC50 of each chemical compound on PBMC by the numerical value of IC50 of the same compound on K562 cells of SI unit, which is shown in the table above, this amounts indicate the selected behavior of the chemical and suggest it as a good option for treatment.

Discussion

It has been shown that abnormal tyrosine kinase activity is one of the causes abnormal cell proliferation in this disease. The effects of pyridol (2 and 3D) pyrimidine on the k562 cell line showed that pyrimidine-derived compounds can inhibit tyrosine kinase activity and the growth and proliferation of these tumor cells (12). In leukemia treatment regimens, peripheral blood mononuclear cells are simultaneously affected by chemotherapy drugs (13). These cells contain natural killer (NK) lymphocytes that are distinct from B and T lymphocytes as their function in tumor cell kill is not dependent on clonal proliferation (14). Due to

this, the inhibitory effects of the pyrimidine compound in the same dilutions tested on the K562 tumor cell line were also affected on PBMC cells, showing the inhibitory effects and reduced survival on PBMC. Cytotoxic effects of hepsulfam and its derivatives have been shown; these agents can induce DNA damage in L1210 leukemia cells (15). The 1,7-heptanedol disulfanate chepsulfan, NS(329680), an alkylating agent with close structural similarity to busulfan, was shown to inhibit the growth of p388 and L1210 mouse leukemia (16). While this index compared to busulfan drug shows a poorer performance of this pyrimidine derivative than busulfan, because of lowering IC50 increases and cytotoxicity. Besides, for the last 20 to 30 years, the major use of the alkylating agent busulfan has been to treat CML (17). Due to higher levels of IC50 of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine on PBMCs regarding the effects of this compound on the cancer class k562 and the numerical values of the SI index, this compound can be used in the treatment of cancer. The combined data of these experiments can be interpreted to imply that the administration of 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine may be a useful agent for preventing cancer. In this disease, it has been proven that it has an abnormal tyrosine kinase activity and causes it to be present in cell proliferation. Inhibition of tyrosine kinase activity in tumor cells inhibits the growth and proliferation of these cells (18). According to the information obtained, we find that the tested compound (4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine)
decreased tumor cell survival by an concentration-dependent manner at all three incubation times of 24, 48 and 72 hours on both k562 and PBMC. Increasing the concentration of the novel tested pyrimidine derivative reduced the survival of K562 class tumor cells. Today, one of the therapeutic approaches to treat cancer is therapeutic differentiation or the use of differentiating agents. As leukemia cells stop at different stages of their differentiation, the idea emerged that cancer could be controlled and treated using drugs that cause these cells to differentiate and eventually lead them to death. Therefore, by inducing spontaneous cell death, the likelihood of cancer recurrence is reduced. Also the use of therapeutic differentiation agents along with chemotherapy causes most cancer cells to become sensitive to the drug. On the other hand, K562 cells, which are considered an in vitro model for acute phase chronic myeloid leukemia, can differentiate into various blood types including erythroid, macrophage, monocyte, and megakaryocyte by different compounds. For example, Hemine induces erythroid differentiation (19), 12-Tetracanvil-furyl-13-acetate (TPA) induces monocyte differentiation (20), and phorbol dibutyrate and phorbol 12-myristate-13-acetate induce megakaryocyte differentiation in the K562 cell line (21). AO/PI Dual staining is a very simple, fast and accurate way to distinguish apoptotic cells from necrotic ones. Necrotic cells have an orange swollen nucleus (22). Data obtained from light and fluorescent microscope observations, as well as DNA fragmentation tests in the present study, indicated the occurrence of apoptosis in K562 cells treated with 4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine. These data showed that the cells treated with these compounds shrunk along with their nuclei, and by DNA fragmentation inside the nucleus, apoptotic bodies are formed, which confirms the occurrence of apoptosis in the cells. Compared to healthy cells, primary apoptotic cells shrink in the form of light green particles in the nucleus and secondary apoptotic cells appear as orange apoptotic bodies (20). According to these and the results of morphological changes related to apoptosis and DNA fragmentation test in treated K562 cells (at IC50 concentration) after 48 hours, it can be said that the test compound (4-chloro-5-iodo-7H-pyrrolo[2.3-d]pyrimidine) in this research can differentiate K562 cells into programmed cell death and prevent cancer cell proliferation from an apoptotic pathway.

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