Article

Selenylated imidazo[1,2-*a*]pyridine induces cell senescence and oxidative stress in chronic myeloid leukemia cells

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Abstract: Imidazo[1,2-*a*]pyridines have been studied regarding drug development. The objective of this work was to evaluate the antileukemic capacity of imidazo[1,2-*a*]pyridine derivatives by screening its ability as a pro-oxidant. Imidazo[1,2-*a*]pyridine derivatives were synthesized and oral bioavailability and toxicity were analyzed in silico. Redox screening was performed on human Kasumi, KG-1, K562 and Jurkat leukemia cells. The imidazo[1,2-*a*]pyridine derivative and the most responsive leukemic cell were selected for cytotoxicity, cell proliferation, cell senescence and oxidative stress assays. The predictive toxicity analysis showed a possible effect on the reproductive system, but without mutagenic, carcinogenic or irritability effects. MRK-107 against K562 cells was the compound that showed the best redox profile. MRK-107 did not induce cell death in K562 and monocyte cells. However, this compound was able to decrease cell proliferation and increase cell senescence after 48 and 72 hours. Furthermore, MRK-107 induced oxidative stress in K562 cells after 72h, increasing lipid peroxidation and decreasing reduced glutathione (GSH) contents. This study demonstrated that MRK-107-induced senescence with the involvement of oxidative stress as a possible mechanism of action, addressing this compound as a potential antitumor drug against chronic myeloid leukemia.

Keywords: Leukemia; imidazo[1,2-*a*]pyridines; selenide; oxidative stress; senescence; chronic myeloid leukemia.

1. Introduction

Leukemia is a set of malignant disorders that present an excess of white blood cells in the blood and/or bone marrow [1]. There is a range of hematopoietic malignancies currently subcategorized according to their morphology, immunophenotype, cytogenetic and molecular abnormalities, and clinical features [2].

The chronic myeloid leukemia (CML) is a myeloproliferative neoplasm and accounts for 15% of adult leukemias [3]. Since the introduction of imatinib in 2000, the annual mortality has decreased from 10-20% down to 1-2% [3]. Despite the success of this therapy, the prevalence of CML has increased and has been suggested that in 2040 the incidence

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rate and mortality rate will balance [4, 5]. However, the cure of CML is functionally not molecular because imatinib does not act directly on the bases of CML, but competing for the ATP binding site of tyrosine kinase, restoring cell death mechanism [6]. Therefore, it is important to continue research of therapies that increase the rate of complete answer and that improve the eradication of minimal residual disease, with fewer side effects and less toxicity.

Compounds containing imidazo [1,2-*a*] pyridines have been largely used in medicinal chemistry and drug development because these compounds are correlated with many therapeutic properties, including anticancer [7], anti-inflammatory [8], antidiabetic [9] and antimalarial [10]. Owing on the pharmacological importance of imidazo[1,2-*a*]pyridines and biological relevance of organochalcogenides, the molecular hybridization of these two moieties results in some interesting therapeutic properties [11-13]. The properties of imidazo [1, 2-*a*] pyridine derivatives have been related to an important strategy in drug research as novel chemotherapeutic agents, because they can increase the anticancer effect in less toxic and equally effective doses, taking the DNA and cell death [14].

Imidazo[1,2-*a*]pyridine proved to be potent P3IK/mTOR inhibitors with excellent kinase selectivity, inducing cell cycle arrest and apoptosis [15]. On the other hand, organoselenium compounds modulate many biological processes, including oxidative stress, overgeneration of reactive oxygen species (ROS), mitochondrial disfunction and DNA damage [14,16]. Considering the pharmacological importance of imidazo[1,2-*a*]pyridine and the biological importance of organoselenides, molecular hybridization of this two moieties results in certain therapeutic properties, and recently there were reported the potential antitumor effect of selenylated imidazo[1,2-*a*]pyridines, in such way showing promising activity on breast cancer cells [11], hepatocarcinoma cells [12], with cytotoxicity, inhibition of proliferation, apoptosis, and selectivity of tumor cells at the relatively low micromolar range. These effects were related to oxidative damage and inhibition of proteins involved in cell proliferation pathways.

As imidazo[1,2-*a*]pyridines induce oxidative stress in tumor cells [11,12], we hypothesized their involvement in the antiproliferative mechanism by inducing senescence of leukemic cells. Cell senescence is a programmed arrest of growth preventing cell proliferation [17]. Thus, when apoptosis does not induce cancer cell death, the induction of cell senescence is an alternative for tumor suppression [18].

Thus, in relation to our continuing interest in the biologically-relevant organoselenium compounds and functionalization of imidazo[1,2-*a*]pyridines [19-26]., the main goal of the present research is the search of new drugs for treatment of myeloid chronic leukemia. Considering that oxidative stress, induction of apoptosis, and senescence are important process involved in response of cancer cells therapy, we used imidazo[1,2-*a*]pyridine and its chalcogen derivatives in leukemic cell lines to investigate its antitumor effects with interest on a potential chemotherapeutic activity against the CML.

2. Results and Discussion

The parameters of oral biovailability and toxicity evaluated using the SwissADME software and Osiris® Property Explorer respectively, are shown in **Table 1**. According to this analysis only one presented violation of the Lipinsk rules [27].

Table 1. Theoretical parameters of oral bioavailability and toxicity of compounds.

Compound	MRK-107	MRK-113	MRK-115	MRK-116

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mLogP	3,37	3,76	2,54	4,61
MW (g/mol)	393,34	363,31	208,26	316,42
n° of violations	0	0	0	1 (mLogP > 4,15)
Mutagenicity	-	-	-	-
Tumorigenicity	-	-	-	-
Irritability	-	-	-	-
Effects on reproduction	Yes	Yes	Yes	Yes
Absorption in the GIT	High	High	High	High

Note: MW: molecular weight; GIT: gastrointestinal tract

According to Lipinski and colleagues (1997) these rules are related to the molecular properties that are necessary for the studied compound to present good physicochemical characteristics such as solubility, intestinal permeability and oral bioavailability [27]. Therefore, only the compound MRK-116 can present problems with solubility, oral bioavailability and consequently absorption, because of a mLogP higher than the ideal value of 4,15. All the other compounds showed promising oral bioavailability.

In addition, about the toxicity all the compounds presented low risk for all the parameters evaluated, such as mutagenicity, tumorigenicity, irritability and effects on reproduction. Therefore, all the compounds were considered promising in the *in silico* studies. Consequently, in vitro studies were initiated to evaluate the antileukemic potential of imidazo[1,2-*a*]pyridines.

The generation of ROS is necessary for normal cell function, but it may also be suggested in cancer therapy. Therefore, cell lines from leukemic models were incubated with imidazopyridine compounds to assess the potential of these compounds to induce intracellular ROS generation. Cells were exposed to the same concentration of 10 μM and analyzed by fluorescence. The cells used in the screening were Jurkat (Acute lymphocytic leukemia-*a*LL), K562 (MCL), KG1 (Acute Myeloid Leukemia -*a*ML) and Kasumi (AML) (Figure 2).

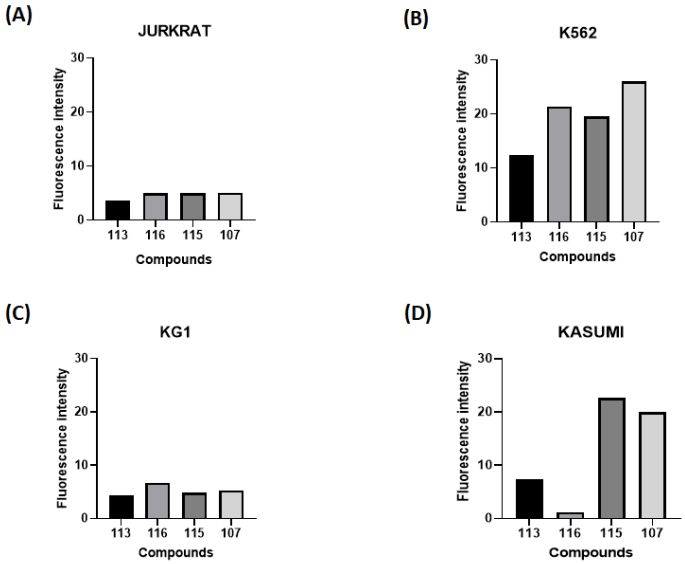

Figure 1. Fluorescence intensity obtained in different leukemic cells (Jurkrat, K562, KG1 and Kasumi) after treatment with Imidazo[1,2-*a*]pyridines (10 μM).

As can observed in **Figure 1**, the cell line K562 (**1B**) showed the highest fluorescence intensity, as well as the highest rate of intracellular ROS, notably the marker 107. The application of ROS in the therapeutic field of hematopoietic cancer cells has been linked to a

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therapeutic mechanism, as anticancer drugs induce an increase in ROS generation, leading cancer cells to apoptosis or senescence [28,29]. Thus, the K562 and MRK-107 cell lines were selected for the following assays, as the MRK 107 was the imidazo[1,2-*a*]pyridine compound that showed the best oxidative profile.

In the cell viability assay, MRK-107 was tested at different concentrations in monocytes. MRK-107 at concentrations of 10, 50, and 100 μM/mL presented cell viability of 89.93, 86.66, and 87.53%, respectively (**Figure 2A**), indicating that MRK-107 did not induce cytotoxicity in any tested concentrations.

The assay was performed to assess cell viability and to evaluate the cytotoxic and proliferative effects induced by MRK 107, which could eventually lead to cell death. The cells K562 were stimulated with the MRK-107 for 24, 48 and 72 hours and analyzed by flow cytometry (**Figure 2**B). The test demonstrated that the administration of MRK-107 was not time-dependent, as cell viability was not significantly affected, suggesting that cell death by apoptosis did not occur. However, the proliferation assay (**Figure 2**C) showed that after 72 hours there was a significant reduction in cell count, demonstrating that MRK 107 induced an inhibition of cell proliferation.

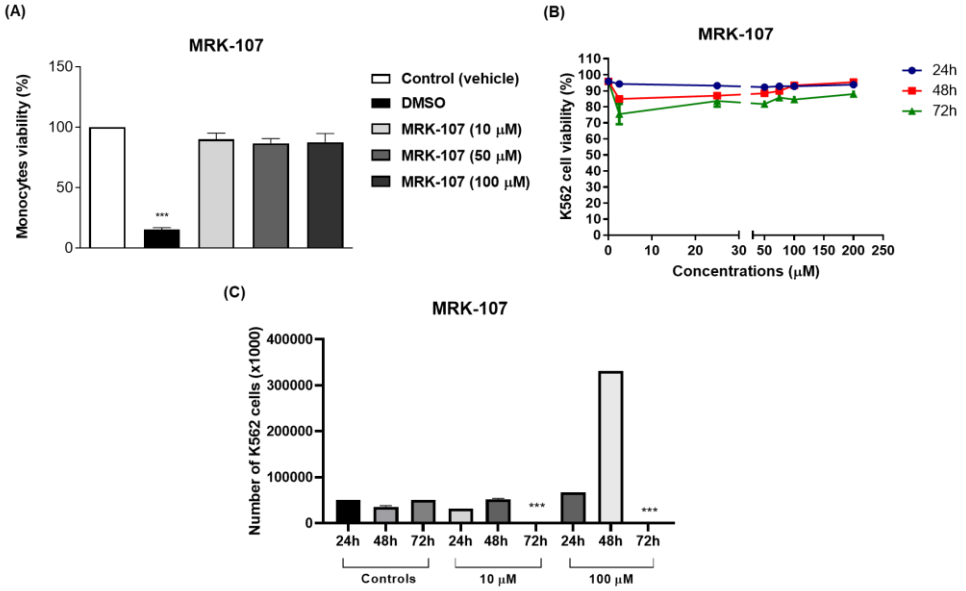

Figure 2. Effect of treatment with MRK-107 at different concentrations on cell viability and proliferation. **(A)** Percentage of monocyte cell viability; **(B)** Percentage of viability of K562 cells after MRK-7 treatment at 24, 48, and 72 hours; **(C)** Cell counts after MRK-7 treatment at 24, 48, and 72 hours. (***) $p < 0.001$ compared to the control group.

The results obtained corroborated those of Santos and collaborators who, through the analysis of a compound derived from selenylated imidazopyridine, obtained a significant reduction in cell counts when compared to cells not treated with the compound, that is, they reported that imidazopyridine derivatives have an antiproliferative potency in the cells of glioblastoma [30]. Another study by Almeida and collaborators using the same imidazopyridine-derived compound, demonstrated an inhibition of cell growth in 90% of breast cancer cell line when compared to untreated controls [11]. Another study carried out by Santos and collaborators also evaluated the oxidative damage and the antiproliferative effect of selenylated imidazo[1,2-*a*]pyridines in hepatocarcinoma cell lines and obtained a reduction in the proliferative capacity of 80% after 72 hours, that is, also corroborating that compounds derived from imidazopyridines induce a state of cell death [12]. Therefore, the study was continued to evaluate the mechanism of senescence.

The percentage of senescent cells in 48 and 72 hours (**Figure 3**) at two non-toxic concentrations of MRK-107 was significantly higher than 50%, which indicates that the

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administration of this compound is able to induce cells to senescence, thereby being a desired and consistent effect for chemotherapy.

It is well known that senescence is one of the tumor suppressor mechanisms and that it causes an irreversible interruption of cell proliferation and that this condition can be induced in response to chemotherapy [31-33]. Senescence is a mechanism that causes the cell cycle to stop in order to potentially inhibit cell cycle progression and consequently allow the proliferation of dysfunctional or transformed cells to occur [34,35].

The mechanisms that cause senescence are broad and include ROS overgeneration, DNA-damage, mitochondrial disfunction [36], while the use of radiotherapy and chemotherapy drugs for example, is known as "therapy-induced senescence" [34]. Guo and collaborators (2010) published that a treatment with hydrogen peroxide (H_2O_2), which induces oxidative stress, resulted in 80% of mouse embryonic fibroblasts to become senescent, which corroborates the present study, since MRK 107 showed a high rate of intracellular ROS in K562 cells, as well as a significant higher rate of senescent cells after treatment with the respective marker [37]. Accordingly, Zhong and collaborators (2019) in a study with breast cancer cells, also demonstrated that oxidative stress mediated by H_2O_2 treatment also caused an induction of senescence in these cells [38].

In addition, a review study on hydroxyurea (HU), an antineoplastic drug widely used in neoplastic and non-neoplastic conditions, reported that the compound is able to reduce cell proliferation as it can induce a state of cell senescence [39]. According to some related studies, HU promotes a deficiency of proteins that regulates oxidative stress, thus contributing to the elevation of ROS and, consequently, to the establishment of cellular senescence [40-42]. These findings are well in line with the data obtained in the present study, as they indicate that increased levels of ROS contribute to cellular senescence. In such a way, senescence may be a protective mechanism against tumor growth, which prevents an uncontrolled proliferation of cancer cells or cells that contain some oncogene activation or the loss of tumor suppressor genes, corroborating some other related studies that also support senescence as a proliferation suppressor mechanism that can stop tumor growth [43,44].

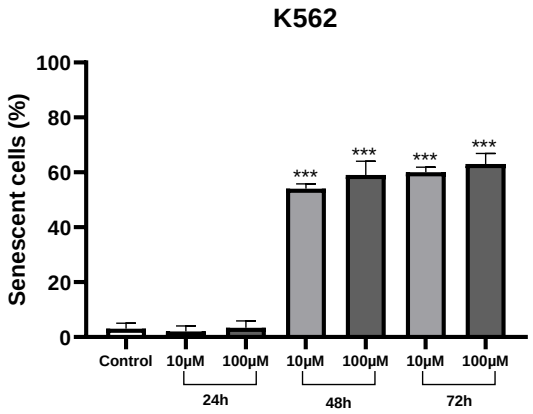

Figure 3. Percentage of senescent K562 cells after 24, 48 and 72 hours of MRK-107 incubation (10 and 100 μM). (***) $p < 0.001$ compared to the control group.

Through the analysis of TBARS and reduced glutathione (GSH) in 72 hours, it was possible to observe the presence of oxidative stress after stimulation of the K562 cell with MRK-107 (**Figure 4**). TBARS (nmol/ml) is a product of lipid peroxidation of the cell membrane, which occurs in the presence of oxidative stress, while GSH is a naturally consumed antioxidant in the presence of oxidative stress, thus increasing TBARS (**Figure 4A**) and decreasing GSH (**Figure 4B**) indicate the presence of oxidative damage induced by MRK-107.

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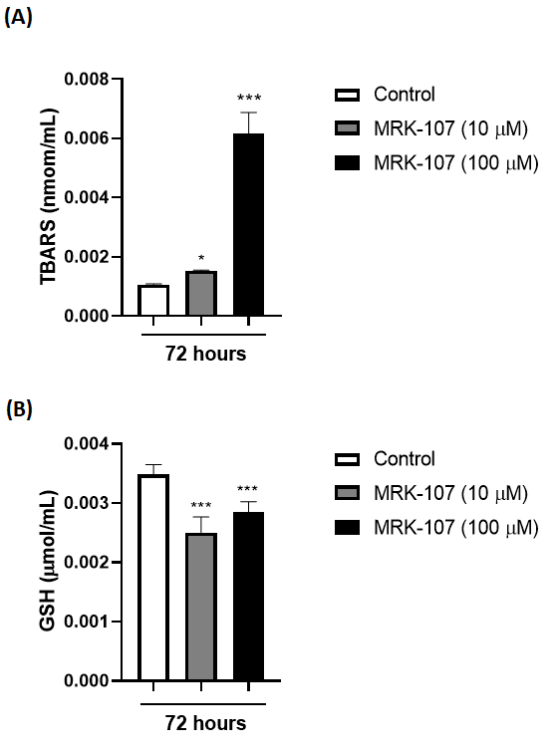

Figure 4. Oxidative stresse markers in K562 cells **(A)** Amount of TBARS (nmol/ml) after stimulation with MRK-107 (10um and 100um) in 72 hours. **(B)** Amount of reduce gluthatione - GSH (umol/ml) after stimulation with MRK-107 (10 uM and 100 uM) at 72 hours. (*) $p < 0.05$ compared to the control group; (***) $p < 0.001$ compared to the control group.

The studies of cell damage associated with senescence are still difficult to detect, however several studies have studied the most toxic forms with more immediate effects, such as DNA damage. DNA breakage is considered one of the most toxic forms of cell damage, one of its immediate consequences include cell cycle arrest, that is, senescence as it is known that telomere shortening and chromosomal instabilities are well-established factors for senescence. In addition, studies report an increase in oxidized intracellular compounds, such as lipofuscin and carbonyl, glycated and modified proteins by lipid peroxidation in aged senescent cells[45].

These data corroborate other findings in the literature as well, in which a low level of GSH in the brain of mice was found associated with a detoxification of endogenous toxins originated through cellular lipoperoxidation processes arising from an insufficient removal of H_2O_2 that favors the production of a oxidative stress [46]. In addition, in that same study, a higher density of senescent cells was found associated with mice with the brain more exposed to oxidative stress, demonstrating that oxidative stress is an important factor that causes senescence [46].

4. Materials and Methods

4.1 Synthesis of imidazo[1,2-*a*]pyridines and chalcogenated derivatives

The starting material, imidazo[1,2-*a*]pyridine MRK-115, was synthesized by refluxing 2-*a*mino-4-methylpyridine 4 and bromoacetophenone 5 in ethanol for 4 h [47]. A series of chalcogenated imidazo[1,2-*a*]pyridine (MRK-107, MRK-113, MRK-116) were synthesized through C(sp2)-H bond selenylation/sulfenylation of imidazo[1,2-*a*]pyridines MRK-115 using diorganyl diselenides/disulfides (**Figure 5**), as previously described by us [48-51].

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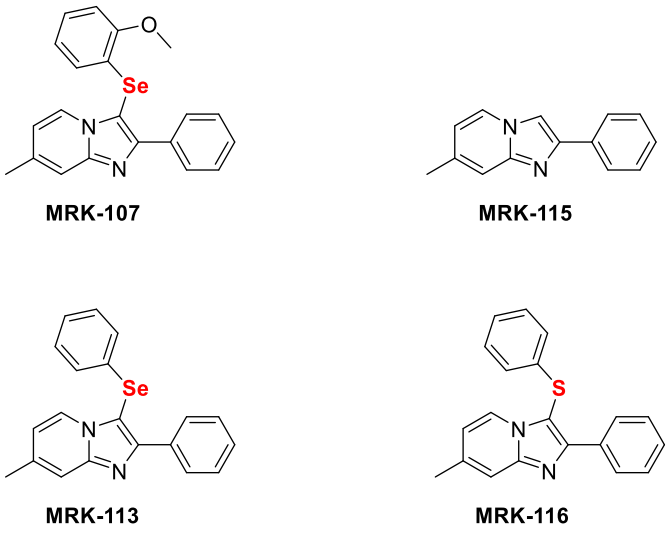

Figure 5. Chemical structure of the imidazo [1,2-*a*] pyridine and its chalcogen derivatives, used in this study.

4.2 Oral bioavailability and toxicity of compounds: *in silico* analysis

The oral bioavailability of the compounds was evaluated using the SwissADME software, an online tool developed by the Swiss Institute of Bioinformatics (SIB) which is available at http://www.swissadme.ch/ that predictively evaluates the pharmacokinetics, based on the similarity to medicines and the medicinal chemical compatibility of compounds. Compounds were evaluated according to criteria established by Lipinski et al. (1997) [27] and Veber et al. (2002) [52], namely: mLogP, molecular weight (MW), number of hydrogen bond acceptors (N and O atoms), number of hydrogen bond donors (NH and OH radicals), number of rotatable bonds and area of polar surface (TPSA).

Toxicity tests were performed using Osiris® Property Explorer, a free program that, by analyzing the chemical structure of compounds, shows relevant properties of drugs and possible drugs. The properties evaluated were: mutagenicity, tumorigenicity, irritability and negative effects on reproduction.

4.3 Cell Culture

Human leukemic cell lines (Kasumi-1, KG-1, K562, and Jurkat) were obtained from American Type Culture Collection (ATCC). KG-1 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum (FBS) (Cultilab, Brazil). The other lineages were maintained in Roswell Park Memorial Institute (RPMI 1640) (Sigma Aldrich, Germany) medium supplemented with 10% FBS. All cells were cultured in medium containing 100 U/mL penicillin (Sigma-*a*ldrich, Germany) and 100 μg/mL streptomycin (Sigma-*a*ldrich, Germany) and in a humidified incubator containing 5% CO_2 at 37°C. The passage numbers for all leukemic cell lines were between 3 and 6.

Peripheral blood mononuclear cells were obtained from three healthy donors (human monocytes). All human volunteers provided informed consent. Separation of mononuclear cells was performed by gradient centrifugation methods using Ficoll Histopaque-1077 (1.077 g/cm^3) (Sigma-Aldrich) following the manufacturer's instructions. The use of human samples was approved by the local Ethical Committee of the Universidade Federal de Mato Grosso do Sul (CAAE35853720.2.0000.0021). The cells were maintained in IMDM supplemented with 20% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere at 37 °C in 5% CO_2.

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4.4 Redox effect screening: measurement of intracellular ROS

Initially, the screening of imidazo[1,2-*a*]pyridines and leukemia cells was performed based on the redox effect. Then, the leukemic strain most responsive to treatments with the compounds (best oxidative profile) was selected for the following stages of the study. Intracellular ROS content were evaluated as reported by [53]. Human leukemic cell lines were incubated for 12 h with imidazo[1,2-*a*]pyridines (10 μM), washed twice with HBSS, and then 100 μl of HBSS/well was added. After, the cells were loaded with DCFH-DA (10 μM) in HBSS at 37 °C and incubated for 30 min. Excess DCFH-DA was removed by washing with fresh HBSS. The intensity of fluorescence was measured at 485 nm for excitation and 530 nm for emission using a Multiscan microplate reader (Thermo Fisher Scientific Oy®).

4.5 Cytotoxicity assay

The MTT assay was performed according to Mosmann (1983) [54] and using human monocytes. Briefly, monocytes were dispensed in 96-well culture plates and placed in an incubator at 37 °C, 5% CO_2. After 24 h, MRK-107 (10, 50, and 100 μM/mL, 100 μL) was diluted in IMDM medium and added. As a negative control, cells were maintained without stimuli. After 48 h, the supernatant was removed, 10 μL of MTT solution (5 mg/mL) were added to the remaining cells, and the plate incubated for 2 h. The medium containing MTT was removed and lysis solution (200 μL of DMSO) was added to each well and homogenized. After 20 min, the absorbance was read at 540 nm in an ELISA reader (HumanReader HS). The results were expressed as percentage values (%) of viable cells compared to the control group.

4.6 Cell death assay

K562 cells were plated (10^5 cells/mL) and stimulated with the MRK-107 (0-200 μM) for 24, 48 and 72 h. After this period, the cells were washed and resuspended in the buffer solution (0.01 M HEPES, pH = 7.4, 0.14 M NaCl, and 2.5 mM CaCl2). The suspensions were labeled with propidium iodide (PI) (Becton Dickinson) according to the manufacturer's instructions. The cells were incubated at room temperature for 20 min. A total of 10,000 events were collected per sample. Flow cytometry evaluation was performed in a flow cytometer. Data were analyzed using the FlowJo software (Tree Star Inc).

4.7 Cell proliferation assay

K562 cells were incubated with MRK-107 at concentrations of 10 and 100 μM for 24, 48 and 72 h. Cell counting was performed using a Neubauer chamber [55]. The growth constant was calculated using the logistic growth equation.

4.8 Senescence assay

Induction of senescence was assessed by measuring SA- β-galactosidase (β-Gal) activity using the senescence cells cytochemical method was conducted as described by Dimri et al. 1995 [56. K562 cells were plated (10^5 cells/mL) and stimulated with the MRK-107 (10 and 100 μM) for 24, 48 and 72 h. After, the cells were washed in PBS and incubated at 37°C (no $C0_2$) with fresh senescence associated stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml [stock = 2mM $MgCl_2$, 5 mM $K_4Fe(CN)_63H_2O$, 5mM, $K_3Fe(CN)_6$ PBS buffer, pH 7.4]. Cell staining and morphology were assessed by microscopy after 12 hours (microscope: Leica®). Results were expressed as percentage of senescent cells.

4.9 Oxidative stress markers

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Oxidative stress markers were evaluated in K562 cells treated with MRK-107 compound (10 and 100 μM) for 72 hours [57]. The cells were homogenized in a cold buffer containing 20 mM sodium phosphate, pH 7.4, 0.1% Triton and 150 mM NaCl (48×106 per 600 μL), except for the reduced glutathione assay that was homogenized in a TCA 12% solution. The determinations were performed using the supernatant after centrifugation (5000g for 5 min).

4.9.1 Lipid Peroxidation Assessment

The endogenous lipid peroxidation was evaluated in the tests by detection of thiobarbituric acid reactive substances (TBARS), particularly malondialdehyde (MDA), according to the method described by Bird and Draper (1984) [58]. Briefly, the homogenate was precipitated with 12% TCA, followed by incubation in bufer (60 mM Tris–HCl, pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid) and 0.73% thiobarbituric acid, at 100 °C, for 60 min. After cooling, the samples were centrifuged (5 min at 10,000g) and the absorbance of the chromophore was measured at 535 nm. The results were expressed in nmol TBARS/mL.

4.9.2 Reduced glutathione assay (non-protein thiols)

The reduced glutathione (GSH) contents were determined from acid extracts in 12% TCA, using the reagent DTNB (5.5′-dithiobis-2-nitrobenzoic acid), according to Beutler et al. (1963) [59]. After being centrifuged at 5000g for 5 min, the supernatants from the acid extracts [12% TCA, 1:10 w/v] were added to 2.5 mM DTNB in 0.2 M sodium phosphate buffer, pH 8.0, and the formation of the yellow thiolate anion was immediately measured at 412 nm. Determinations were expressed in μmol/mL.

4.10 Statistical Analysis

The results were expressed as the mean±standard deviation (SD). The data were analyzed using two-way ANOVA followed by the Bonferroni or Tukey-Kramer test. Values of were considered statistically significant. Statistical analysis was obtained from three independent experiments and was performed by GraphPad Prism software, version 8.0 (San Diego, USA).

5. Conclusions

In conclusion, this study demonstrated that the senescence induced by the compound MRK-107 has the involvement of oxidative stress as a possible mechanism of action, being a potential antitumor mechanism in the chemotherapy of cancer cells using compounds derived from imidazo [1,2-*a*]pyridines against the CML.

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Sample Availability: Samples of the compounds are available from the authors.

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