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ORIGINAL ARTICLE

Anticancer and Anti-Inflammatory Effects of Benzothiazole Derivatives Targeting NF-κB/COX-2/iNOS in a Hepatocellular Carcinoma Cell Line

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ABSTRACT

Objectives: Benzothiazole compounds, characterized by their diverse biological and pharmacological properties, have emerged as promising molecules for suppressing cancer cell proliferation and invasion due to their antiproliferative attributes. Prior research from our laboratory revealed that 2-substituted benzothiazole compounds inhibit the proliferation of glioma and cervical cancer cells and induce apoptosis in pancreatic cancer cells. However, there is limited research on the effectiveness of benzothiazoles against hepatocellular carcinoma cells (HCC). This study sought to elucidate the anticancer potential of 2-substituted benzothiazole derivatives through their modulation of oxidative stress and inflammation mediators.

Materials and Methods: Antiproliferative effects of two-step synthesized 2-substituted benzothiazole derivatives were evaluated on HepG2 cells via MTT assay. Apoptosis induction was assessed using Annexin V/PI staining; cell cycle arrest effects were determined through cell cycle analysis; cell migration was examined via wound healing assay; and mitochondrial membrane damage was quantified using JC-1 staining. Spectrophotometric measurements of total antioxidant status (TAS), total oxidant status, superoxide dismutase (SOD), total thiol, and native thiol levels were used to assess cellular redox status. Expression of nuclear factor kappa B (NF-κB), an inflammatory marker, was assessed by western blot, while inflammation-related cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase levels were measured using ELISA.

Results: This investigation unveiled benzothiazole derivatives' antiproliferative and cytotoxic properties against HepG2 cells (IC_{50} values of 56.98 μM and 59.17 μM at 24 h, and 38.54 μM 29.63 at 48 h). The synthesized compounds exhibited the ability to suppress cell migration and induce apoptosis, mediated by mitochondrial membrane potential loss (wound-closure rates of 84.0 and 90.4% vs. 51.7% control at 48 h, apoptosis rates of 10.70% and 45.22% vs. 1.02% control). Furthermore, these derivatives reduced SOD activity (A and B at 100 μM $p<0.001$), TAS levels (A and B at 100 μM, $p < 0.05$, $p < 0.001$), and dynamic disulfide content. Notably, a decrease in NF-κB protein levels, closely associated with inflammation, was observed, along with a subsequent reduction in downstream effectors COX-2 (A and B at 100 μM, $p<0.001$) and iNOS (A and B at 100 μM, $p < 0.001$).

Conclusion: The findings of this study underscore the antiproliferative effects of benzothiazole derivatives in human HCCs, coupled with their anti-inflammatory potential by diminishing NF-κB levels.

Keywords: Benzothiazole, cyclooxygenase-2, inflammation, inducible nitric oxide synthase, nuclear factor kappa B, oxidative stress

INTRODUCTION

Hepatocellular carcinoma (HCC), a primary liver cancer, arises from a complex interplay of factors, including viral hepatitis, fatty liver disease, alcohol consumption, and the ingestion of aflatoxin-containing foods, ultimately resulting in liver inflammation, cirrhosis, fibrosis, and aberrant regeneration.[1] Among these factors, chronic hepatitis B and C virus infections inflict cellular damage, chronic alcohol consumption triggers steatohepatitis, and obesity fosters triglyceride accumulation, alters the hepatocyte microenvironment, and incites inflammation.[2]

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Nuclear factor kappa B (NF-κB) is an essential transcription factor in regulating cell proliferation, survival, and inflammation.[3] Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are the primary enzymes involved in the inflammatory response, and their expression levels are elevated in response to NF-κB activation.[4] The increased production of these enzymes leads to an accumulation of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS), which exacerbate inflammation, disrupt redox homeostasis, induce DNA damage, and promote cellular transformation toward malignancy.[5] Given the intimate link between cancer development and pro-inflammatory factors, a primary objective in pharmaceutical chemistry is to devise cost-effective synthetic compounds that mitigate the molecular consequences of inflammation and restrain tumor cell proliferation.[6]

Benzothiazoles, whose diverse biological activities are dependent on the substituents and structural arrangement within their heterocyclic nucleus, have garnered significant interest in synthetic and pharmaceutical chemistry.[7] Our previous investigations revealed that benzothiazole compounds with 2-substituted groups can induce apoptosis in pancreatic adenocarcinoma cells (PANC-1), reduce cell viability, and decrease antioxidant levels.[8] This highlights the potential therapeutic effectiveness of these compounds. In addition, we observed that these compounds have antiproliferative effects in cervical cancer and glioma cells.[9,10] Given these positive results, understanding how 2-substituted benzothiazole compounds may affect inflammation and oxidative stress modulation in HCCs has become particularly important, shedding light on their potential therapeutic effects in liver cancer treatment. Due to their diverse biological roles, encompassing anticancer, anti-inflammatory, antimicrobial, and anticonvulsant properties, benzothiazole derivatives have emerged as subjects of keen exploration.[9-12] Clinical trials have scrutinized benzothiazole-containing compounds for neurological and psychiatric disorders, affirming their therapeutic potential.[11,13,14] Furthermore, select benzothiazole derivatives, distinguished by their anticancer prowess, have undergone clinical assessment as chemotherapeutic agents against recurrent melanoma, exhibiting promising results during phase I clinical trials.[15]

While there exists a body of research highlighting the utilization of benzothiazoles in the development of new drugs for various ailments, the anticancer attributes of 2-substituted benzothiazole derivatives against hepatocellular carcinoma, their interplay with inflammation, and the molecular mechanisms governed by the NF-κB/COX-2/iNOS signaling pathway remain incompletely understood. In light of this, the present study endeavors to elucidate the mechanisms underlying the anticancer and anti-inflammatory effects of synthesized 2-substituted benzothiazole derivatives on HCC.

MATERIALS AND METHODS

Synthesis of 2-substituted benzothiazole derivatives

The synthesis of 2-substituted benzothiazole compounds (A and B) was performed in two steps, following previously established protocols.[8-10,12] Chalcone analogs were synthesized via an aldol condensation reaction in the first step. Specifically, 4-fluorobenzaldehyde or 4-nitrobenzaldehyde (2.3 mmol) was reacted with cis-bicyclo[3.2.0]hept-2-en-6-one, in a basic medium containing sodium hydroxide (NaOH, 2.3 mmol) at room temperature. This reaction yielded chalcone analogs. In the second step, the chalcone analogs (1 mmol) were refluxed with 2-amino-thiophenol (1 mmol) in the presence of a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH) to produce benzothiazole compounds (Figure 1). Chloroform was added to the reaction mixture, and the organic phase was washed, dried with sodium sulfate, and filtered. The final products (A and B) underwent purification via chromatography, using hexane mixed with a gradually increasing concentration of ethyl acetate (0-15%). This procedure resulted in a solid compound with a waxy consistency. After purification, the compounds were kept under cold storage conditions (-20 °C). The structure of the compounds was explained based on spectroscopic methods (NMR, IR, and elemental analysis), and comparisons with authentic samples.[8-10]

Cell culture and cell viability assay

Culturing, passaging, incubation of cells, and preparation of compounds were conducted as defined previously.[16] Cell proliferation was measured using the MTT test, one of the most commonly used cell viability tests. HepG2 and L929 cells were seeded and treated with compounds A and B at concentrations of 10, 25, 50, 75, and 100 μM. Sorafenib, which is used in the treatment of HCC in the clinic, was also applied to HepG2 cells as a positive control at concentrations of 0.1, 0.5, 1, 5, and 10 μM. After treatment, the medium was removed from the plates containing the compounds, and the MTT solution (0.5 mg/mL, Sigma) was added to the cells. After incubating the plates for 4 hours, dimethyl sulfoxide was added. At the end of this period, the absorbance values of the samples at 570 nm and 650 nm wavelengths were measured.

Apoptosis assay

The Annexin Apoptosis kit (BD Biosciences) assessed apoptosis in HepG2 cells.[17] Compounds A and B were applied at 100 μM doses in experiments exclusively designed to induce apoptosis. After incubation for 48 hours, the cells were subjected to trypsinization and rinsed in phosphate-buffered saline (PBS). Subsequently, the cells were suspended in a binding buffer, and 5 μL of fluorescein isothiocyanate (FITC) and propidium iodide (PI), dyes, were mixed in the suspensions. A Beckman Coulter flow cytometer was used to evaluate samples within 15 minutes, with the Kaluza Analysis software (Beckman Coulter) employed to analyze the data.

Cell cycle test

The Propidium Iodide Flow Cytometry Kit (Abcam, ab139418) was used to study the cell cycles.[18] HepG2 cells were treated with 100 μM compounds A and B for 48 hours. After trypsinization, washing, and suspension in PBS, the cells were kept in a mixture of PBS and ethanol at 4 °C for at least two hours. Next, they were resuspended in PBS and stained with PI.

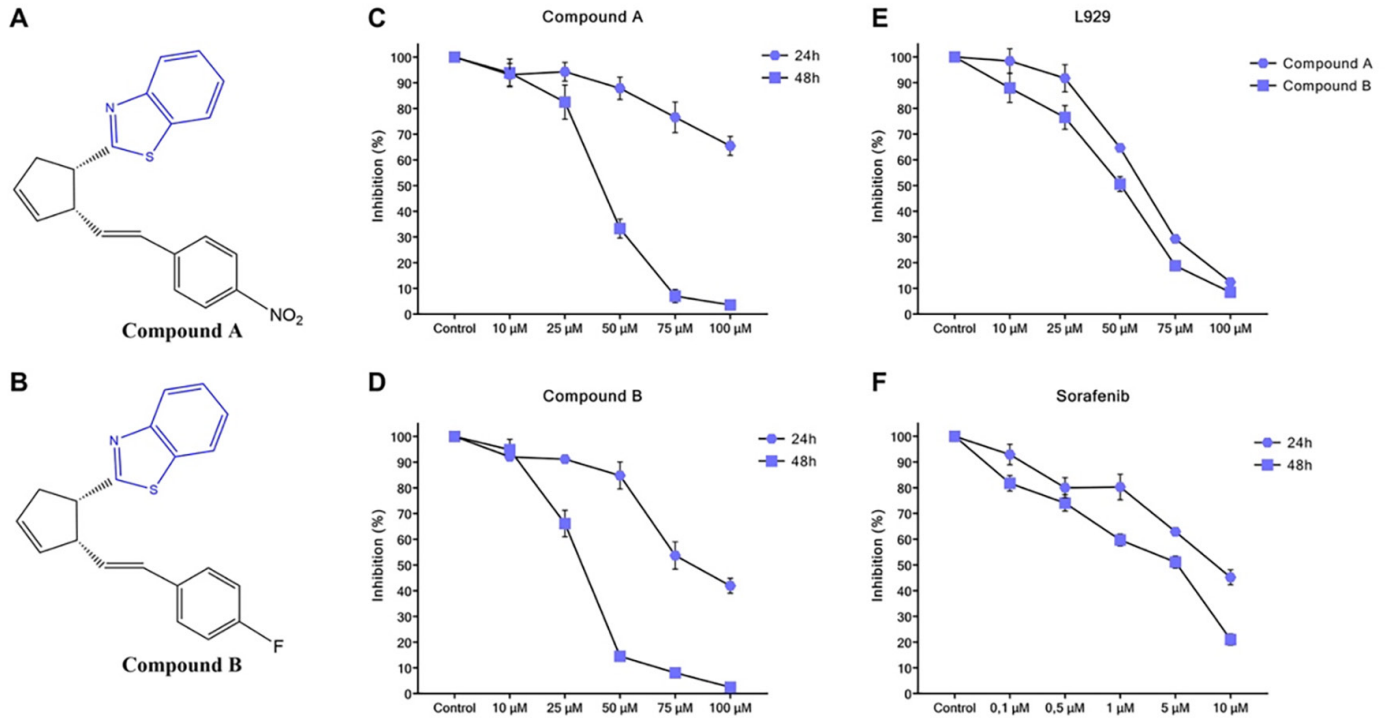

Figure 1. Synthesized 2-substituted benzothiazole compounds and inhibition effects of A and B on HepG2 and L929 cell lines

(A, B) In the initial step, chalcone derivatives were synthesized by combining cis-bicyclo[3.2.0]hept-2-en-6-one with benzaldehyde (4-nitro or 4-fluoro) compounds in the presence of NaOH. Subsequently, the chalcone compounds obtained in the second step were subjected to a reaction with 2-amino-thiophenol in the presence of p-toluene sulfonic acid, forming benzothiazole compounds.

The inhibition rates of A and B administrations on HepG2 and L929 cell lines were evaluated. The control groups, where no drug administration took place, showed an inhibition rate of 0%. (C) Cell viability of compound A in HepG2 cell line at 24 and 48 hours. (D) Cell viability of compound B in HepG2 cell line at 24 and 48 hours. (E) Cell viability of compounds A and B for 48 hours in the normal cell line (L929). (F) Cell viability of compound Sorafenib in HepG2 cell line at 24 and 48 hours

The data were analyzed using Kaluza Analysis software after examining the samples with a Beckman Coulter flow cytometer.

Mitochondrial membrane potential assay (ΔΨM)

Once the HCCs had reached confluence in a 96-well plate, they were treated with various doses (50, 75, and 100 µM) of compounds A and B. This was done by adding the compounds directly to the cell culture media. The cells were then incubated for 48 hours to allow the compounds to take effect. Subsequently, 100 µL of a JC-1 solution (Abcam, ab113850-JC1) was added to each well.[19] The plate was incubated at 37 °C for 10 minutes in the dark. A fluorescent microscope was used to capture an image of the plate with a Texas red filter, with excitation/emission at 590/610 nm.

Wound healing analysis

The Ibidi brand wound healing culture apparatus was used to evaluate wound width.[20] Cells were seeded in split wells, and the apparatus was removed after confluency. Zero-time images (T0) of the cells were photographed under the microscope. Cells were photographed again 48 hours (T48) after administering compounds A and B (50, 75, and 100 µM). Cell migration rates were determined by evaluating the gap closure after A and B administration.

Western blot analysis

Protein analysis by Western blot was performed as previously described in.[21] Briefly, analyses were performed in supernatants obtained from cells lysed with Radioimmunoprecipitation Assay (RIPA) buffer. Samples subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis were electrophoresed and transferred to the polyvinylidene difluoride membrane. The bands were imaged and analyzed after incubation with the appropriate primary and secondary antibodies.

Oxidative stress parameters and COX-2 and iNOS activity

Compound A and B concentrations (50, 75, and 100 µM) were used to treat both types of cancer cells and the cells were allowed to incubate for 48 h. The culture medium was removed from the incubated cells, and the cell pellets were lysed. After centrifuging the mixture at 14,000 × g for 10 min, the supernatant was obtained and used to measure oxidative stress parameters. Total antioxidant status (TAS), total oxidant status (TOS), and total and native thiol concentrations in the supernatant were determined using a spectrophotometer (microplate reader, Synergy H1). The TOS to TAS ratio was acknowledged as an oxidative stress index (OSI) at a fixed ratio. The OSI=TOS/TAS formula was used to calculate the OSI value. The activity of superoxide dismutase (SOD) was measured by assessing the decrease of nitroblue tetrazolium generated by xanthine and

xanthine oxidase according to the procedure described by Sun et al.[22]. COX-2 and iNOS were measured by an ELISA kit according to the manufacturer's procedure (SunRed Biotechnology).

Statistical Analysis

The statistical analyses used GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Shapiro-Wilk tests were used to assess the normality of the data. One-way ANOVA was used for group statistical comparisons, followed by Dunnett's multiple comparison tests.[23] If the data did not follow a normal distribution, the Kruskal-Wallis and Dunn's post-hoc tests were used. The data were expressed as means and standard deviations. A p-value less than 0.05 was considered significant and denoted with * for $p < 0.05$, ** for $p < 0.01$, or *** for $p < 0.001$.

RESULTS

Cytotoxic effects of 2-substituted benzothiazole compounds

To assess the cytotoxic effects of compounds A and B on hepatocellular carcinoma, we determined the percentage viability of HepG2 cells. It was evident that the proliferation of hepatocellular cells decreased in a dose-dependent manner when exposed to 10, 25, 50, 75, and 100 µM of compounds A and B, as well as 0.1, 0.5, 1, 5, and 10 µM of the positive control, sorafenib. Notably, the inhibitory properties of these compounds varied, with compound B (a benzothiazole derivative with a fluorine substituent) displaying higher inhibitory activity compared to compound A (a benzothiazole derivative with a nitro substituent), particularly at the 24-hour time point (Figure 1C, D).

Although significant inhibition of the benzothiazole compounds was observed at the 48-hour time point, with 100 µM of compound A inhibiting HepG2 cell growth by 96.4%, 100 µM of compound B exhibiting a growth inhibition of 97.5%, and 10 µM of sorafenib displaying a 79% growth inhibition, further investigation is necessary to understand the mechanism of inhibition. When evaluating the obtained MTT data, the IC50 values for compound A were determined to be 56.98 µM after 24 hours and 38.54 µM after 48 hours, respectively. The IC50 values for compound B after 24 hours and 48 hours were 59.17 µM and 29.63 µM, respectively.

To further evaluate the antiproliferative effects of these compounds, we conducted experiments using the L929 mouse fibroblast cell line. In normal cells (L929), the IC_{50} concentrations for compound A and compound B at 48 hours were 53.84 µM and 40.16 µM, respectively (Figure 1E, F).

Effects of 2-substituted benzothiazole compounds on apoptosis

The apoptotic cell ratios were assessed following the treatment of HepG2 cells with 100 µM compounds, which exhibited significant cytotoxic effects. The apoptotic cell population was compared to the control group after administering benzothiazole compounds to the cells for 48 hours. In the control group, the apoptotic cell rate was 1.02%. However, following treatment with 100 µM concentrations of compounds A and B, the apoptotic cell rates were 10.70% and 45.22%, and the necrotic cell rates were 48.70% and 23.49%, respectively (Figure 2).

Effect of 2-substituted benzothiazole compounds on the cell cycle

Our research on the effects of compounds A and B, which are 2-substituted benzothiazole compounds, on the cell cycle has promising implications for cancer treatment and drug development. Through our analysis of the distribution of cell cycle phases in PI-labeled cells, we discovered that a significant proportion of HepG2 cells treated with these compounds at a concentration of 100 µM were in the sub-G1 phase. This indicates apoptosis and DNA fragmentation. After treatment with compounds A and B, the percentage of the cell population in the sub-G1 phase was found to be 42.23% and 55.53%, respectively (Figure 3). These findings suggest that the compounds have the potential to be used in cancer therapy and drug design.

Effect of 2-substituted benzothiazole compounds on mitochondrial membrane potential

The impact of 2-substituted benzothiazole compounds on ΔΨm was evaluated using a cationic dye called JC-1 and fluorescence microscopy. The control group exhibited intense red fluorescent staining, indicating the aggregation of JC-1 dye within the mitochondria. To assess the effect of benzothiazole compounds, concentrations of 50, 75, and 100 µM were applied; the red fluorescence intensity of JC-1 dye was analyzed. It was observed that both substituents A and B caused a dose-dependent reduction in the red fluorescence intensity (Figure

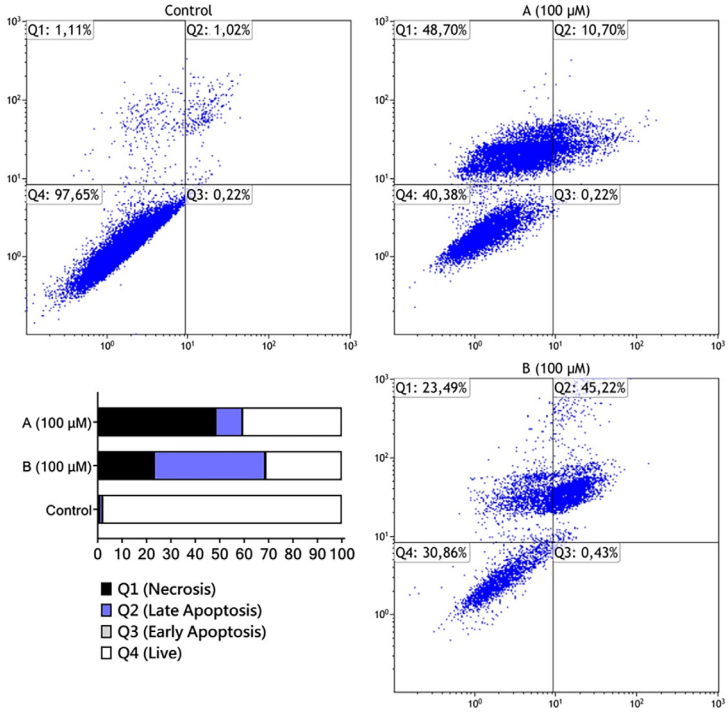

Figure 2. Annexin V flow cytometry analysis of the effects of A and B on HepG2 cell line. 100 µM concentration of A and B can cause apoptosis. FITC-Annexin V was utilized to evaluate apoptosis in HepG2 cells following a treatment period of 48 hours with 100 µM each of compounds A and B. The total number of Annexin V+/PI+ quadrants reflects the degree of apoptosis

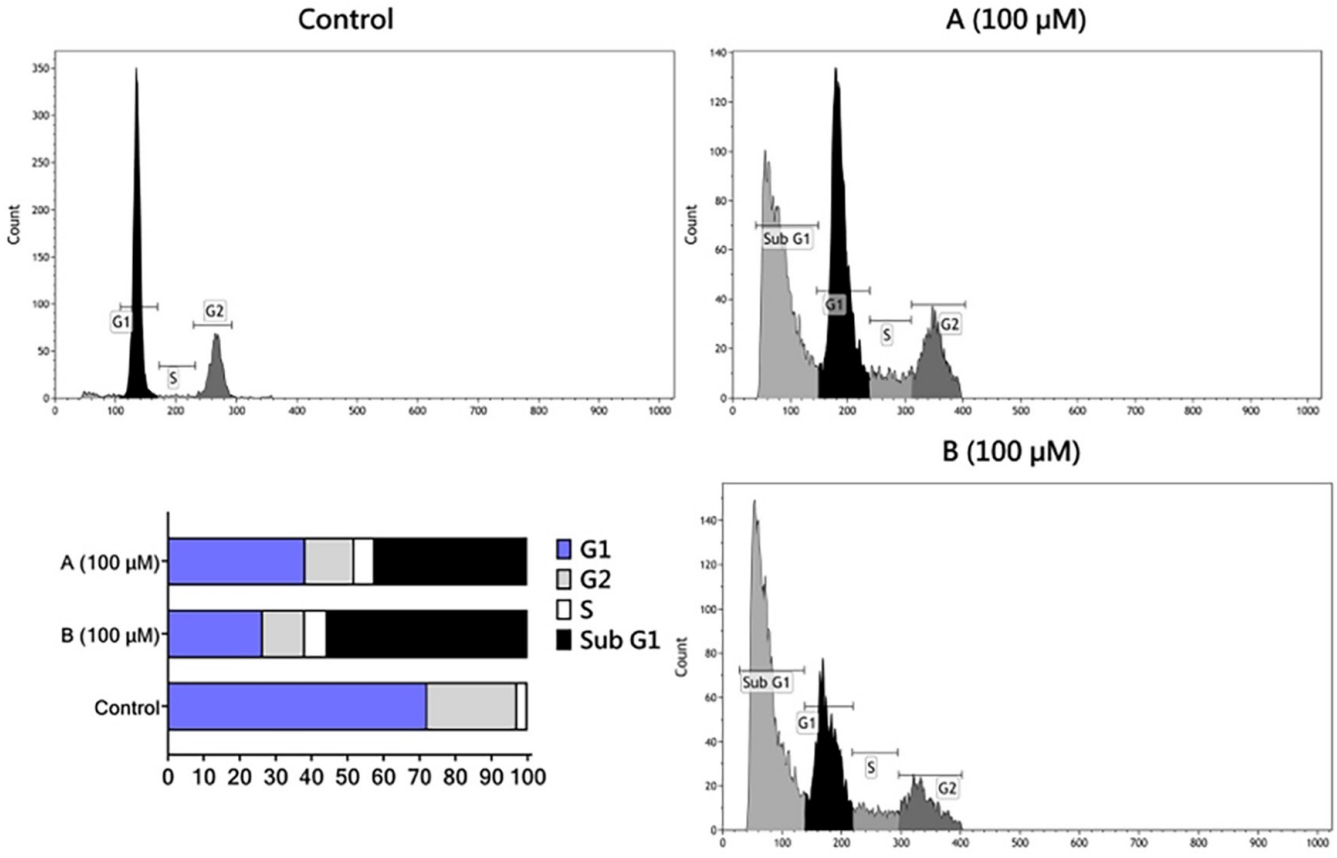

Figure 3. Effects of A and B on cell cycle. The effects of A and B on the cell cycle of HepG2 cells were examined. Administration of 100 µM doses of A and B each caused the cells to shift to the sub-G1 phase

4). These findings suggest that 2-substituted benzothiazole compounds induce apoptosis by disrupting the ΔΨm.

Effect of 2-substituted benzothiazole compounds on wound healing

To assess the influence of 2-substituted benzothiazole compounds on the migration of HepG2 cells, we evaluated T0, T24, and T48 images to measure changes in wound widths. The wound width of HepG2 cells after 48 hours was 51.7% in the control group. In contrast, treatment with 50 and 100 µM of compound A resulted in wound widths of 80.6% and 84.0% in HepG2 cells, respectively, while compound B led to wound widths of 89.2% and 90.4%, respectively. These observations indicate that 2-substituted benzothiazole compounds effectively suppress cell migration in a dose-dependent manner (Figure 5).

Effects of 2-substituted benzothiazole compounds on NF-κB, COX-2, and iNOS

The inflammation and cell proliferation processes in cancer formation are intricately linked to the effects of inflammatory mediators, including NF-κB activation, COX-2, and iNOS, on transcriptional regulation.[24] Consequently, we evaluated NF-κB, COX-2, and iNOS levels to investigate the underlying inflammatory mechanisms influenced by 2-substituted benzothiazole compounds in liver cancer.

When HepG2 cells were treated with 50 and 100 µM concentrations of compounds A and B, we observed a dose-dependent decrease in NF-κB protein expression levels (Figure 6A, B). The Western blot results underscore the anticancer effects of 2-substituted benzothiazole compounds, highlighting their role in mediating a reduction in NF-κB protein expression levels, a pivotal mediator of inflammation.

We also scrutinized the impact of benzothiazole compounds on COX-2 and iNOS enzymes, which are critical players in the inflammatory process. Our observations indicated that 2-substituted benzothiazole compounds, when applied to HepG2 cells, led to a dose-dependent inhibition of COX-2 and iNOS (Figure 6C, D). These findings suggest that compounds A and B effectively reduce COX-2 and iNOS levels by operating downstream from NF-κB inhibition and consequently mitigate heightened inflammation associated with carcinogenesis.

Effect of 2-substituted benzothiazole compounds on oxidative stress

Previous studies have demonstrated that drugs employed in cancer treatment induce cell death by either causing cellular damage or disrupting the redox balance, through oxidative stress mechanisms in tumor cells.[25] Consequently, in this study, we assessed the levels of, TOS, TAS, OSI, SOD, total thiol, and native thiol to elucidate the pro-oxidant effect of 2-substituted benzothiazole compounds on HepG2 cells.

In HepG2 cells treated with doses of 50, 75, and 100 µM of compounds A and B, TAS, SOD, total thiol, and native thiol

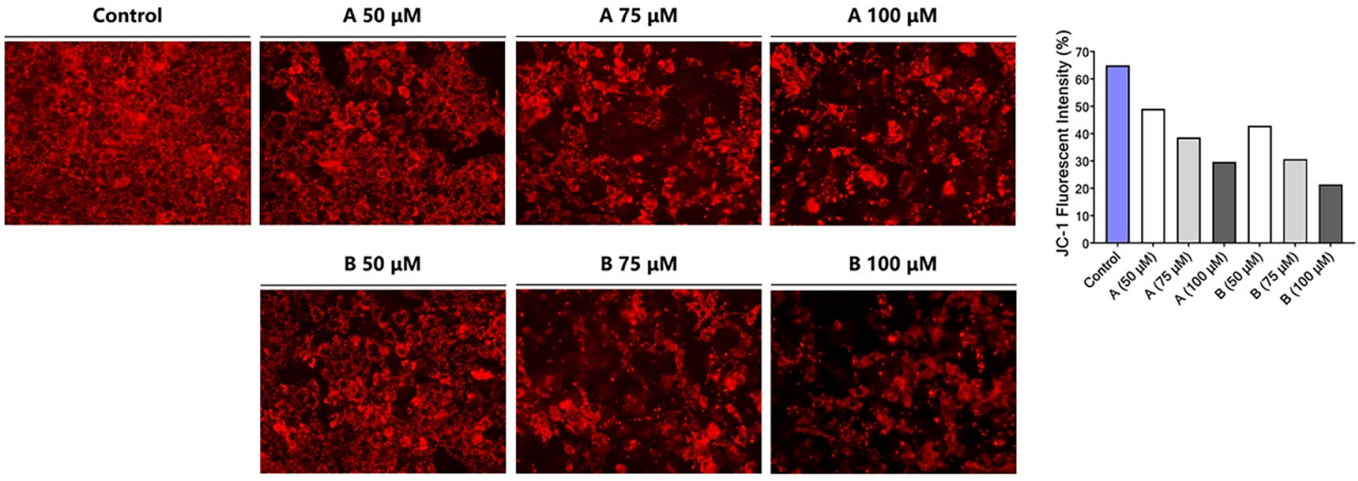

Figure 4. Effect of A and B on mitochondrial membrane potential. A red fluorescence image of HepG2 cells stained with JC-1 was taken after administering varying concentrations of compounds A and B, with each at concentrations of 50, 75, and 100 µM. The aggregation of JC-1 dye in cells decreased in a dose-dependent manner with the administration of A and B

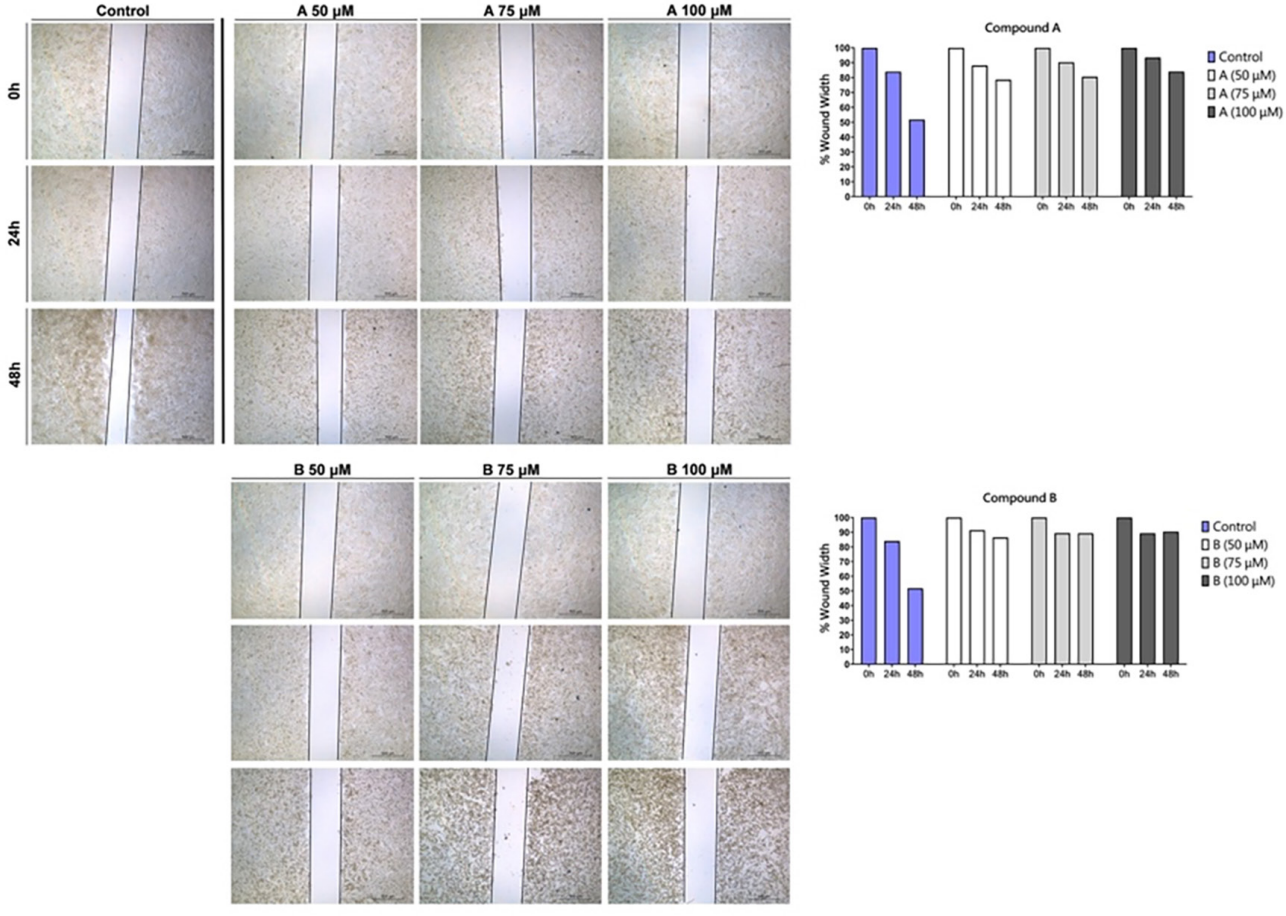

Figure 5. Effect of A and B on wound healing in HepG2. Images of wound width in cells treated with A and B at 0, 24, and 48 hours. Treatment with benzothiazole derivatives decreased the closure of wound width in cells in a dose-dependent manner

levels exhibited a dose-dependent decrease (Figure 7A, D-F). Notably, the most pronounced increase in TOS level and OSI was observed at a concentration of 100 µM (Figure 7B, C). These findings indicate that the elevated oxidant levels induced cell death in HepG2 cells through the actions of 2-substituted benzothiazole compounds.

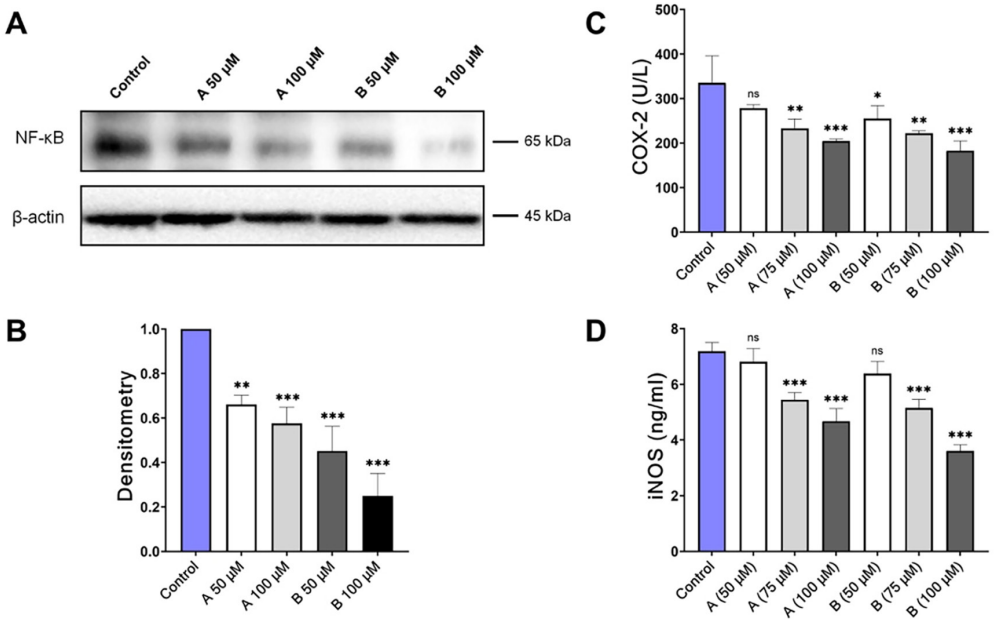

Figure 6. Effects of A and B on NF-κB protein expression and COX-2 and iNOS activity. Western blot bands showing NF-κB protein expression levels after administration of A and B at 50 and 100 µM doses (A). Densitometric analysis of NF-κB protein levels (B). Different concentrations of compounds A and B affect COX-2 (C) and iNOS (D) levels in HepG2 cells

NF-κB: Nuclear factor kappa B, COX-2: Cyclooxygenase-2

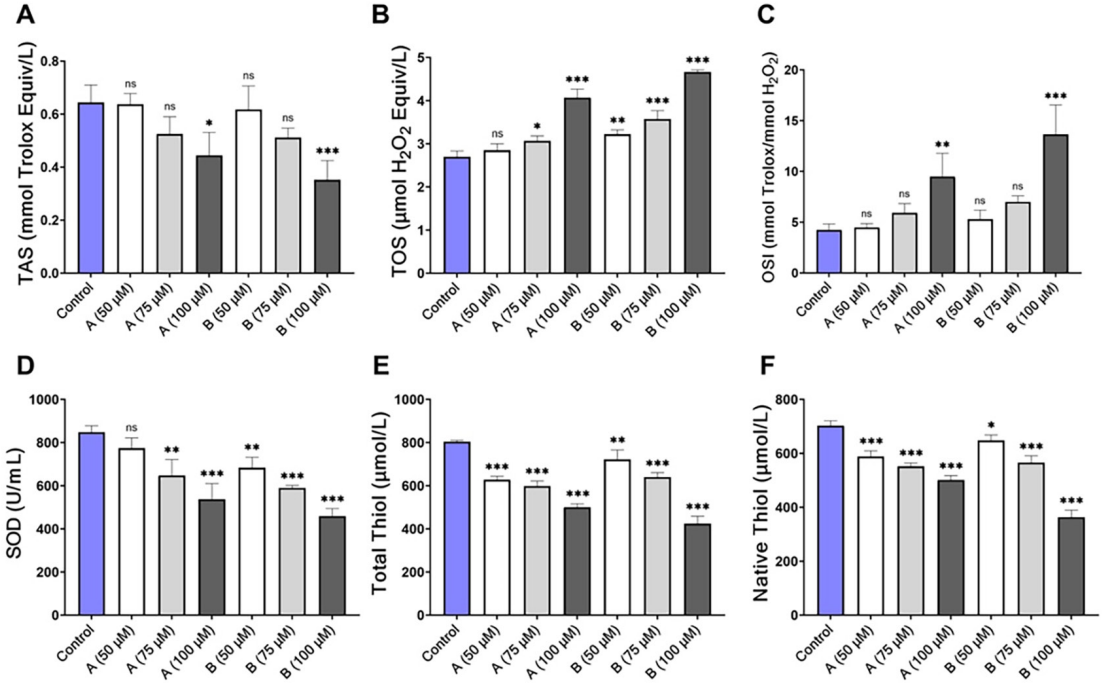

Figure 7. Effect of A and B on oxidative stress parameters in HepG2. HepG2 cells were treated with 50, 75, and 100 µM of A and B for 48 hours; TAS (A), TOS (B), OSI (C), SOD (D), total thiol (E), and native thiol (F) levels were measured

TAS: Total antioxidant status, TOS: Total oxidant status, OSI: Oxidative stress index, SOD: Superoxide dismutase

DISCUSSION

HCC is a significant health problem worldwide, leading to impaired control of fundamental processes such as cellular differentiation, proliferation, and metastasis.[26] In our study, we evaluated the antiproliferative and anti-inflammatory effects of 2-substituted benzo[d]thiazole derivatives on HepG2 cells and demonstrated that these effects are mediated through the NF-κB/COX-2/iNOS signaling pathway. Our findings align with previous studies supporting the potential of synthetic compounds with similar structures to suppress proliferation and target inflammation-related signaling pathways in different cancer types.[27] The cost-effective production and development of synthetic compounds; their easy accessibility; low toxicity; high selectivity; and potential for combination therapy offer significant advantages in the fight against cancer.[28] However, despite extensive research on the molecular mechanisms of signaling pathways in hepatocytes associated with liver cancer, chemotherapeutic approaches to target these signals remain limited.

Our study aimed to assess the impact of 2-substituted benzothiazole compounds on the proliferation of hepatocarcinoma cells. We employed a concentration range of 10 to 100 μM for our experiments. Following a 24-hour treatment with compounds that contained nitro (A) and fluorine (B) substituents, we determined IC_{50} values of 56.98 μM and 59.17 μM, respectively. Notably, we observed that benzothiazole compounds with both substituents exhibited similar effects in suppressing the proliferation of HepG2 cells. This discovery implies that 2-substituted benzothiazole compounds possess inhibitory properties against hepatocarcinoma cell growth. Furthermore, our findings indicate that benzothiazole compounds have a more significant inhibitory effect on HepG2 cancer cells than healthy mouse fibroblast L929 cells. This observation suggests that these compounds may exhibit selectivity in their action, offering a promising avenue for further investigation in developing targeted cancer therapeutics.

The induction of apoptosis and cell cycle arrest are two critical mechanisms commonly employed to elucidate the ability of anticancer drugs to inhibit tumor growth.[29] Numerous studies have demonstrated that benzothiazole-based derivatives induce apoptosis in vitro in various cancer types, such as prostate, stomach, lung, breast, colon, and glioblastoma.[30] Modi et al.[31] reported that a phenyl derivative benzothiazole compound initiated caspase-dependent apoptosis in cervical cancer (SiHa) cells and enhanced the tumor suppressor effect of the p53 protein. Peng et al.[32] demonstrated that a small molecule, 2-substituted benzothiazole compound, increased DNA fragmentation in human melanoma cells' sub-G1 phase of the cell cycle (A375). Li et al.[33] discovered that a 2-substituted benzothiazole compound mediated apoptosis induction in B lymphoma cells (Ramos) by arresting the cell cycle in the G0/G1 phase, inducing loss of ΔΨm, and elevating caspase-9/8 and caspase-3 protein levels. In line with previous studies, our flow cytometry analysis of AnnexinV/FITC and PI-labeled cells revealed an increase in the percentage of apoptotic cells and an accumulation of cells in the sub-G1 phase with increasing concentrations of compounds A and B in HepG2 cells. Additionally, the red fluorescence signal of JC-1 dye within the cells was progressively diminished in a dose-dependent manner. These findings suggest that benzo[d]thiazole derivatives may induce apoptosis through ΔΨm loss and exhibit anticancer effects in HepG2 cells.

NF-κB is a pivotal nuclear transcription factor that regulates gene expression in the cell cycle. It plays a crucial role in controlling normal cellular functions and contributes to the onset of diseases such as cancer. Viral infections (such as hepatitis B or C) and liver tissue inflammation lead to sustained NF-κB activation.[34] This prolonged activation promotes cell proliferation, angiogenesis, and invasion and simultaneously inhibits apoptosis. Understanding the regulation of NF-κB and its impact on COX-2 and iNOS enzymes is vital for cancer research.[35] Therefore, our study aims to investigate the impact of synthesized 2-substituted benzothiazole derivatives on NF-κB protein expression and COX-2 and iNOS enzyme inhibition, which are critical factors in inflammation and cancer progression. Previous research suggests that the ability of certain compounds containing a thiazole ring to induce apoptosis and suppress inflammation is associated with the regulation of NF-κB/mTOR/PI3K/Akt signaling activity.[36] However, our study is the first to establish a link between the anti-inflammatory effect of benzothiazole-derived compounds and their potential to inhibit COX-2 and the iNOS enzymes, which are involved in downstream inflammation. Our findings demonstrate that benzothiazole compounds with fluorine and nitro substituents suppress COX-2 and iNOS activation in HepG2 cells by inhibiting NF-κB, subsequently leading to apoptosis through the anti-inflammatory mechanistic signaling pathway. These results suggest that benzothiazole-derived compounds could be a novel therapeutic approach for hepatocellular carcinoma characterized by inflammation.

It is well-known that elevated levels of ROS within cells can function as a mechanism to target cancer cells. The initiation of oxidative stress in cancer cells may prompt programmed cell death via DNA damage, mitochondrial damage, and activation of apoptosis. Consequently, researchers are currently investigating ROS regulators or drugs that can reduce ROS production in cancer cells as a prospective strategy for cancer therapy. Our study assessed the impacts of 2-substituted benzothiazole compounds on HepG2 cells to induce apoptosis by promoting oxidative stress and ROS accumulation. Our findings reveal that compounds A and B diminish the antioxidant defense in hepatocarcinoma cells through reductions in TAS, dynamic disulfide levels, and SOD enzyme activity. Moreover, they induce oxidative stress by elevating TOS and OSI levels. Previous investigations have documented that a benzothiazole core compound can produce cellular damage by inhibiting SOD and glutathione reductase enzymes, thereby increasing oxidative stress in breast cancer.[37] Another study has shown that a water-soluble benzothiazole derivative compound can induce loss of function and cell death by accumulating ROS in B-lymphoma cells.[33] These studies underscore the potential of oxidative stress as a targeted attack mechanism in cancer

cells and propose that benzothiazole-derived compounds might induce damage in cancer cells due to their pro-oxidant effect.

CONCLUSION

This study shows that compounds containing 2-substituted benzothiazole effectively reduce the cell viability of hepatocarcinoma cells. Furthermore, these compounds cause cellular damage, resulting in increased oxidative stress, fragmentation of DNA, and loss of $\Delta\Psi m$. Additionally, these compounds suppress the activation of the inflammation-inducing NF-κB/COX-2/iNOS signaling pathway, which plays a critical role in developing hepatocellular carcinoma. Our study is the first to establish a connection between the apoptotic effects of benzothiazole-derived compounds and the proteins and enzymes NF-κB, COX-2, and iNOS. Moreover, our study sheds light on the inhibitory effects of these compounds on inflammation. These findings offer valuable insights into the potential utilization of 2-substituted benzothiazole compounds in therapy for cancer and inflammation.

Ethics

Ethics Committee Approval: This study was conducted exclusively on established HepG2 and L929 cell lines and did not involve human participants or animal subjects; therefore, ethics committee approval and informed consent were not required.

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: M.M.Ü., N.Ü., M.C., Design: M.M.Ü., N.Ü., M.C., Data Collection or Processing: M.M.Ü., N.Ü., M.C., Y.T., Analysis or Interpretation: M.M.Ü., N.Ü., M.C., Y.T., Literature Search: M.M.Ü., N.Ü., Writing: M.M.Ü., N.Ü.

Conflict of Interest: The authors declare no conflicts of interest.

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