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Novel thiosemicarbazone derivative 17B interferes with the cell cycle progression and induce apoptosis through modulating downstream signaling pathways

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ABSTRACT

Thiosemicarbazones (TSCs) are interesting group of chemical compounds that received significant levels of attention due their wide range of pharmacological effects including antibacterial, antiviral, and especially antitumor activities. Several thiosemicarbazone derivatives have been extensively reported recently with their antitumor properties but designing and developing novel thiosemicarbazone derivatives with more potent chemotherapeutic activities is of great interest for cancer future cancer therapy. Thus, here we aimed to demonstrate as yet undetermined anti-cancer properties of novel thiosemicarbazone derivative 17B. Viability of cells was determined using MTT assay and LDH activities were analyzed using lactate dehydrogenase activity assay. Apoptosis were assayed using Annexin V-FITC and PI double staining method and cell cycle analysis was achieved by using PI staining with fluorescence-activated cell sorting and migration capacities of cells were determined by wound healing assay. As a result, 17B limited cell viability and showed cytotoxic effects in a dose-dependent manner in A549, MCF7 and U2OS cells. In addition, it inhibited progression through cell cycle by interfering with the G1/S transition and triggered apoptosis by modulating expression levels of pro-apoptotic and anti-apoptotic mediators in MCF7 and U2OS cells. Also, 17B significantly impaired the migration of cancer cells and delayed wound healing in all cells. Consequently, findings of the present study have strongly indicated that 17B might be a novel anti-cancer agent for the treatment of breast cancer and osteosarcoma but not for lung cancer. Our results have provided mechanistic insights into anti-cancer properties of a novel thiosemicarbazone derivative 17B.

Keywords: Anti-cancer agent, Cancer, Cytotoxicity, Thiosemicarbazone Derivative, 17B

INTRODUCTION

According to recent findings of Global Cancer Statistics, it was estimated that 18.1 million new cancer cases and 9.6 million cancer related deaths will occur in 2018 (Bray et al., 2018). Also, lung cancer was identified to be the most frequently encountered type of cancer with a ratio of 11.6 % (among all cases) and responsible from the 18.4 % of the all cancer-related deaths (Bray et al., 2018). Female breast cancer was identified to be the second mostly diagnosed cancer after lung cancer. Additionally, it was shown that the most commonly diagnosed cancer and the leading cause of cancer death varies widely between countries and within each country depending on the socioeconomic status in both genders (Bray et al., 2018). Moreover, although recent progress in cancer therapy is promising, it is not sufficient enough to fight against cancer. Besides, existing chemotherapeutic agents are not very effective and they have adverse side effects that are very difficult to cope with. Also, patients develop resistance to several chemotherapeutics (Kara et al., 2015). Accordingly, developing novel therapeutic agents with strong, selective anticancer effects and low toxicity are of great interest

Thiosemicarbazones (TSCs) are interesting group of chemical compounds that display a diverse range of biological activities including antitumor, antibacterial, antifungal, anti-amoebic, antiviral and analgesic activities (Afrasiabi et al., 2004; Patel et al., 2013). Also, it was well-reported that thiosemicarbazone derivatives are involved in the inhibition of ribonucleotide reductase, which is a rate-limiting enzyme in DNA synthesis, reducing ribonucleotides to deoxyribonucleotides. Thus, they interfere with the nucleic acid synthesis in cells (Li et al., 2001). Although mechanism of ribonucleotide reductase inhibition was not ascertained at first, now we know that metal chelating activities of thiosemicarbazones are the chief factor in their antineoplastic effects (Kalinowski et al., 2009). Furthermore, recent studies have indicated that increased activity of ribonucleotide reductase leads to the destruction of hemostatic balance of normal cells and speeds up malignant transformation (Yu et al., 2009). The thiosemicarbazone derivative compounds, which are ribonucleotide reductase antagonists, exhibit antineoplastic activity by inhibiting DNA synthesis and repair (Alvero et al., 2006). Another activity of thiosemicarbazone derivatives is to render topo-II DNA complex unstable by alkylating thiol residues, showing anticancer activities (Liu et al., 1992; Ferrari et al., 1999). These substances bind to DNA by producing an electrophilic alkyl cation that can react with free electron pairs or nucleophilic centers carrying negative charges. Thus, making covalent bond with DNA and blocking DNA replication. This produces DNA

damage and if the damage is not repaired, the cell cycle is blocked and the cell dies (Liu et al., 1992; Ferrari et al., 1999).

Although antitumor properties and mechanism of the action of several thiosemicarbazone derivatives have been extensively reported, designing and developing novel thiosemicarbazone derivatives with high potential to be used in clinic is of great interest. Accordingly in the present study, we aimed to demonstrate the anticancer activities of a thiosemicarbazone derivative, 17b. Previously, we have shown that 17b have significant anti-proliferative activities in HGC-27 human gastric carcinoma cells in contrast to tested reference antineoplastic agent, Paclitaxel as reported in (Tasdemir et al., 2015). However, the effect of 17b on proliferation, migration and apoptosis of cancer cells remained muchly elusive. Therefore, here we demonstrate significant anticancer activity of a thiosemicarbazone derivative 17b.

MATERIAL AND METHODS

Synthesis of 17b and preparation of solutions

Synthesis of 17b ((S)-(+)-2-[(4-piperidin-1-il)benzilidin])-N-(1-feniletil)hidrazinkarbotiyoamid) ($C_{21}H_{26}N_4S$ (366.52 g/mol)) was achieved as previously described (Karaküçük-İyidoğan et al., 2011; Tasdemir et al., 2015). Briefly, 1 mmol of chiral phenylethylamine was slowly dissolved in 100 ml of chloroform and thiophosgene was added dropwise in a 1: 1 ratio. 3 mmol of sodium hydroxide was dissolved in very small amount of water and added to the reaction medium. After stirring the reaction at 0 ° C overnight, the resulting product was extracted with distilled water and the organic phase was collected and dried with sodium sulfate and purified by column chromatography. The resulting 1 mmol chiral isothiocyanate derivative compound was dissolved in diethylether and 1 mmol hydrazine monohydrate dissolved in the same solvent was added dropwise. The reaction mixture was allowed to stir at room temperature overnight. The resulting viscous product was washed with petroleum ether. 1.53 mmol of 4- (piperidin-4-yl) benzaldehyde was dissolved in 20 mL of methanol and added on 1.53 mmol (S) - (+) - N- [phenylethyl] hydrazinecarbotioamide dissolved in 10 mL of hot methanol. The precipitated solid material was crystallized in ethanol. Synthesized compound (0.40 g, yield; 72 %) was yellow in color and melting point was 79-80 ºC.

Cell culture

In the cell culture experiments, A549 (ATCC CCL-185), MCF-7 (ATCC HTB-22) U2-OS (ATCC HTB-96) cancer cell lines were commercially obtained from ATCC (American Type Culture Collection). Cells were maintained using Dulbecco's modified eagle's medium (DMEM) containing 10 % fetal calf serum (FCS) (all from Sigma-Aldrich, MO, USA) in a humidified carbon dioxide incubator (Thermo Fisher Scientific Inc.,Wilmington, USA) adjusted to following conditions; 37°C and 5 % CO_2. When the cell density in the culture dishes was about 80-90 %, 0.25 % Tripsin-EDTA solution was used to detach cell from the culture plates.

Cell viability assay

To analyze cell viability of cancer cells, colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (from Sigma-Aldrich, MO, USA) assay was used. MTT is a staining technique based on the reduction of mitochondrial enzymes by MTT substance. In this assay, water-soluble MTT dye is converted to insoluble purple formazan particles within cells. Briefly, cells were seeded to 24-well cell culture plates and incubated until they reach 80-90 % confluency. Subsequently, they were incubated with different concentrations (25, 50 ,100 and 200 µM) of 17b for a period of 24 hours. After incubation period, wells were rinsed with HBSS (Hank's Balanced Salt Solution) and treated with 1 mg/ml MTT solution for a period of 45-60 minutes at 37 ºC. Following incubation, MTT was removed and the formazan particles were solubilized with 500 µl DMSO and corresponding concentrations were determined by using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer at 570 nm.

LDH activity assay

For the measurements of the LDH activity in living cells, Lactate Dehydrogenase Activity Assay Kit (#MAK066, Sigma-Aldrich) were used and the manufacturer's recommendations were followed. Briefly, prior to MTT assay, supernatants were saved for the LDH activity assay. Subsequently, for each sample, a total of 50 µl of mixture was prepared with 48 µl of LDH assay buffer and 2 µl LDH Substrate Mix. Then, 50 µl of samples were loaded on 96-well plates and mixed with 50 µl of the prepared reaction mixture. The first reading was performed at 450 nm and readings were repeated every 5 min. Plates were kept at 37 ºC during experiments.

Apoptosis assay

Induction of apoptosis in cells treated with 17b was analyzed by Annexin V-FITC and PI double staining method by using with Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling Technology, #6592) by following the instructions of the manufacturer. Briefly, cells were seeded to 6-well culture plates with a concentration of $1x10^6$ cells per well and incubated overnight and subsequently treated with the 17b. Following 24 hours incubation, cells were detached using Trypsin-EDTA (0.25%) (from Sigma), centrifugated and rinsed with cold HBSS. Cells were later incubated with 12.5 µl PI (propidium iodide) and 1 µl Annexin V for 15 minutes and 140 µl 1X Annexin V Binding Buffer were added prior to analysis. Positive cells were detected and quantified by fluorescence-activated cell sorting (FACS) analysis by using Gallios Flow Cytometer (Beckman Coulter).

Cell cycle analysis

For the analysis of cell cycle, cells were seeded to 6-well culture plates with a concentration of $1x10^6$ cells per well and incubated overnight and subsequently treated with the 17b for 24 hours. After incubation, cells were trypsinized with Trypsin-EDTA and washed with HBSS and subsequently fixed with 70 % cold ethanol solution. Ethanol was removed following fixation and cells were then washed with PBS and centrifugated at 1500 rpm for 5 minutes. Pellet were later resuspended in 200 µl Guava® Cell Cycle Reagent (Guava Technologies, #4500-0220) and incubated in a dark place for 30 minutes at room temperature. Following incubation, analysis was performed in Gallios Flow Cytometer (Beckman Coulter).

Real-time PCR

Isolation of total RNA was achieved by using High Pure RNA Isolation Kit (#11828665001) from Roche with an additional RNase treatment step. Subsequent synthesis of cDNA from obtained RNA samples were achieved by using Maxima H Minus First Strand cDNA Synthesis Kit (#K1681) according to the recommendations of the manufacturer. Sequences and features of the primer sequences used in quantitative amplification reactions were provided in Supplementary Table 1. qPCR were held in Rotor Gene Q (Qiagen Sample & Assay Technologies, Germany) instrument by using Maxima SYBR Green/ROX qPCR Master Mix (#K0221) recommendations of the manufacturer were followed.

Wound healing assay

For the analysis of wound closure, cells were seeded to 6-well culture plates and allowed to form cell monolayer. Subsequently, cells were incubated with the 17b for 24 hours and rinsed with HBSS following incubation. Wounds were then generated using 100 µl pipette tips and washed twice with HBSS to eliminate cell fractionations and first images recorded under light microscope. An image was taken every 24 hours until wounds are closed completely. The gaps were then measured using ImageJ software.

RESULTS

17B restricts cell viability and exerts cytotoxicity in a dose-dependent manner

To observe the inhibitory effects of 17b on viability of A549, MCF7 and U2OS cancer cells and determine the inhibitory concentrations, we used MTT assay in which living cells transform the water-soluble MTT to insoluble purple formazan particles by mitochondrial succinate dehydrogenase as a function of redox potential. Consistent with our previous observations (Tasdemir et al., 2015), treatment with 17b led to a significant inhibition of cell viability in a dose-dependent manner in all three cell lines. Inhibitory concentrations of 17b were determined as 99.8 µM, 213 µM and 203.7 µM in A549, MCF7 and U2OS cells, respectively. DMSO treated group was considered as a control group in comparisons. Furthermore, since lactate dehydrogenase (LDH) is an oxidative enzyme that catalyzes the interconversion of pyruvate and lactate and released as a result cell damage, LDH activity assay provides information about the cellular toxicity in living cells. Accordingly, we further performed LDH assay to measure cellular toxicity upon 17B treatments. In consistent with the anti-proliferative activity of 17B, a dose-dependent elevation of cellular toxicity was observed in cells treated with 17B. Particularly, LDH activities in A549, MCF7 and U2OS cells treated with 17B were 26.23±15.84, 111.3±25.02 and 116.1±22.43, respectively.

17B induces apoptosis and activates caspase-mediated killing

Given that 17B is a novel anti-proliferative agent in A549, MCF7 and U2OS cells, we further wondered whether it induces cellular apoptosis and might be involved in the cellular apoptotic pathways in these cells. To elucidate whether 17B triggers apoptosis in A549, MCF7 and U2OS cells, we used Annexin V-FITC and propidium iodide (PI) double staining with fluorescence-activated cell sorting (FACS) analysis in which translocation of phosphatidylserine was monitored by the help of recombinant annexin V conjugated to green-

fluorescent FITC dye and dead cells using PI. Consistent with the anti-proliferative and cytotoxic activities, 17B significantly triggered cellular apoptosis of MCF7 and U2OS cancer cells as presented in Figure 3. Although apoptosis is also triggered in A549 cells treated with 17B, this change was not statistically significant. Percentages of cells undergoing apoptosis were determined to be 9.40±2.23, 78.25±2.34 and 40.84±1.23 in A549, MCF7 and U2OS cells, respectively. We further wondered whether 17B-induced apoptosis is mediated by the transcriptional inhibition of anti-apoptotic members and activation pro-apoptotic members. To test this possibility, expression levels of BCL2, BCLXL, BAX, CASP3 and CASP9 levels were determined by qPCR. Quantitative analysis of 17B-treated cells demonstrated that 17B-triggered apoptosis is mediated by the transcriptional activation of pro-apoptotic BAX, CASP3 and CASP9 and inhibition of anti-apoptotic BCL2 and BCLXL. Particularly, while the expression levels of BCLXL was significantly downregulated in MCF7 and U2OS cells in a time-dependent manner, it was not significantly altered in A549 cells treated with 17B. In contrast, BCL2 remained unchanged in MCF7 and U2OS cells, but significantly altered in A549 cells treated with 17B in a time-dependent manner. Furthermore, we also tested the activation of pro-apoptotic BAX and caspase-dependent activation of apoptosis in cells treated with 17B. Significant transcriptional activation of BAX was observed in all three cell lines. Consistent with the activation of BAX, expression levels of caspase 3 and 9 was also found to be markedly increased in MCF7 breast cancer cells treated with 17B. Also, expression levels of caspase 3 and 9 was found to be slightly increased in A549 and U2OS cells, yet these changes was statistically insignificant. These findings further supported to notion that 17B targets members of the apoptotic machinery to trigger apoptosis of cancer cells.

17B impairs progression through cell cycle and modulates regulators of cell cycle

Our results thus far have suggested that 17B might induce cell cycle arrest. To elucidate this possibility we further performed cell cycle analysis to observe the effect of 17B in cell cycle arrest by PI staining with fluorescence-activated cell sorting (FACS) analysis in which cells can be quantified according to their DNA content which reflects cell cycle status (G0/G1, S, and G2/M). Consistent with its apoptotic activity, 17B significantly induced accumulation of cells in the G0/G1 phase in MCF7 and U2OS cells, indicating that 17B-mediated killing of cancer cells might be result of DNA damage in these cells. Also, as in the case of apoptosis, 17B did no significant effect to cell cycle arrest in A549 lung cancer cells, further suggesting quite resistance of these cells to 17B-mediated killing. Percentages of cells accumulated in the

G0/G1 phase of cell cycle was determined to be 66.67±2.21, 73.56±2.20 and 80.61±2.2 in A549, MCF7 and U2OS cells treated with 17B, respectively. We further wondered whether 17B-induced cell cycle arrest is a result of the modulation of genes involved in the regulation of cell cycle. To test this possibility, expression levels of CDKN1B, CDKN2B, P53 and CDK2 levels were determined by the help of qPCR. Confirming the findings obtained in the cell cycle analysis, CDKN2B expression levels were markedly elevated as a result of 17B treatments in MCF7 and U2OS cells in a time-dependent manner. P53 and CDKN1B showed a significant increase only in MCF7 cells in a time-dependent manner, indicating the activation of DNA damage response and irreversibly DNA damage in these cells. Also, although differentially expressed in in MCF7 and U2OS cells, expression changes of CDK2 was not statistically significant. In contrast to these findings, consistent with the results of the apoptosis and cell cycle analysis, we obtained controversial findings in A549 cells. While the expression levels of CDK2 was significantly elevated, CDK2B expression was significantly reduced in these cells. These results further suggest that 17B might not be an anti-proliferative agent specific for lung cancer cells. In contrast, it specifically and efficiently targets to eliminate breast cancer and osteosarcoma cells through modulating cellular signaling pathways to induce cell cycle arrest and apoptosis.

17B interferes with the migration of cancer cells

To further reveal the mechanisms of anti-tumorigenic activity of 17B, we wondered whether 17B interferes with the migration of cancer cells and tested migration capacity of cancer cells treated with 17B by using wound healing assay method in which migration of cells are monitored. We found that 17B significantly reduced the migration capacities of cancer cells. In particular, while the wounds in SF and DMSO treated groups were healed at end of the third day, they were closed at the end of the fourth day in 17B treated group in A549 cells. Consistently, in U2-OS cells, while the wounds were completely closed at the end of 24 hours in control groups, they were closed after 72 hours in the 17b group. Similarly, in MCF7 cells, while the wounds were completely closed at the end of fourth day in control groups, they were closed after 9 days in the 17b group. These findings have indicated that 17B interferes with the migratory capacities of cancer cells by restricting their mobility.

DISCUSSION

Because thiosemicarbazones are a well-known class of amine derivatives that have a wide range of pharmacological effects including antibacterial, antiviral, and antitumor activities,

they have gained considerable attention in pharmaceutical industry (da Silva et al., 2010). The mechanisms in which thiosemicarbazone derivatives exert their antitumorigenic properties have been linked to various biological activities including inhibition of ribonucleotide reductase, generation of reactive oxygen species (ROS), inhibition of cell cycle, chelation of metal ions, modulation of cellular signaling pathways regulating cell proliferation and death. Strikingly, several phase I and phase II clinical trials were initiated for thiosemicarbazone derivatives. Findings of the clinical trial studies have shown that thiosemicarbazones alone or in combination with other anti-neoplastic agents such as cisplatin and gemcitabine, interferes with the development and progression of several types of tumors (Mrozek-Wilczkiewicz et al., 2019a).

Accordingly here, we demonstrate as yet undetermined anti-cancer properties of a novel thiosemicarbazone derivative 17B. To explore the mechanism of 17B action on cancer cells, we analyzed the effects of 17B on cell viability, apoptosis, cell cycle and migration capacities of cancer cells. Strikingly, 17B restricted viability of A549, MCF7 and U2OS cells and exerted cytotoxicity in a dose-dependent manner. In line with the anti-proliferative and cytotoxic activities, 17B was also significantly triggered cellular apoptosis of MCF7 and U2OS cancer cells but not A549 lung cancer cells. In addition, pro-apoptotic BAX was activated in all three cell lines and caspases (3 and 9) was only activated in MCF7 breast cancer cells and anti-apoptotic BCLxl significantly downregulated in MCF7 and U2OS cancer cells in a time-dependent manner, indicating specific inhibitory function of 17B in breast cancer and osteosarcoma. Since several anti-cancer drugs such as 5-FU which inhibits DNA synthesis and induces cell cycle arrest at G1/S phase affect the progression of the cell cycle and arrest the cell cycle at different phases, we further confirmed apoptosis findings with cycle analysis and expression analysis of genes involved in the regulation of cell cycle. In consistent with the apoptotic functions of 17B, 17B impaired progression through cell cycle and modulated regulators of cell cycle. Particularly, it was induced cell cycle arrest MCF7 and U2OS but not A549 cancer cells by accumulating cells at G0/G1 phase, indicating that 17B interferes with the G1/S transition. Analysis of genes involved in the regulation of cycle further supported these findings. CDK1B, CDK2B and p53 was shown to be activated in MCF7 cells in time-dependent manner. P53 and CDK2B was also activated after 24 hours of post treatments, their levels were later diminished. As one of the therapeutic actions of anti-cancer drugs is to slow down the migration capacity of cancer cells to prevent the spread of the tumor cells to make metastasis, we analyzed anti-migratory capacity of 17B in all three

cell lines. Our findings were further revealed that 17B significantly interfered with the migration of cancer cells and delayed wound healing. In line with our observations, Rejmund et al. also showed in their study that a novel TSCs containing piperazine interfered with the G1/S transition and resulted in differential expression of PARP, caspase-3, 8, 9, p53, cyclin E, cdc2, p21, and cytochrome c genes in a variety of cancer cell lines (Rejmund et al., 2018). Also, Malarz et al. showed that p21 and cdc2 but not p53 are activated in response TSCs (Malarz et al., 2018). Similarly, thiosemicarbazones based on di-2-pyridine ketone and quinoline moiety was reported to alter expression of cyclin D1, cdc2, p21, p53 and cytochrome c proteins and induce apoptosis in HCT 116 $p53^{+/+}$ and MCF-7 cancer cell lines (Mrozek-Wilczkiewicz et al., 2019b).

In line with our findings, it has been reported that thiosemicarbazone derivatives are potential therapeutic agents in cancer therapy. In particular, Brockman et al. reported that 2-formylpyridine thiosemicarbazone has antileukemic activities in murine animal models (Brockman et al., 1956). Later studies have shown that Triapine, a thiosemicarbazone derivative, exhibits anti-cancer activity in the L1210 leukemia mouse model (Finch et al., 2000). Also, Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), synthesized as a novel iron chelator thiosemicarbazones derivative, has been shown to affect tumor formation and development in various cancers by impairing tumor cell migration and metastasis (Li et al., 2016). Wangpu *et al*. (Wangpu et al., 2016) synthesized two novel thiosemicarbazones derivative, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone, and showed that these compounds suppress cell migration and metastasis by increasing the expression of the NDRG1 (N-myc downstream regulated gene-1) gene in HT29 colon cancer and DU-145 prostate cancer cells. Similarly, pterostilbene carboxaldehyde thiosemicarbazone (PTERC-T) was shown to inhibit cancer cell migration and metastasis (Nikhil et al., 2016). Taken together, in accordance with previous observations, our results have proven that this newly synthesized thiosemicarbazone compound increases the potential for the novel anti-cancer drug because it stimulates apoptosis in cancer cells, stops cell cycle and slows the capacity of cells to migrate.

Moreover, in accordance with our findings, Rejmund et al. showed that TSCs containing piperazinyl fragment have strong anti-cancer properties compared to their previously designed TSC compounds–DpC and Dp44mT. Particularly, this novel TSCs, piperazinylogs of Triapine, was reported to inhibit progression through cell cycle, induce apoptosis and related

gene expression in cancer cells, concluding that Triapine containing piperazine leads to more potent and selective anticancer activity (Rejmund et al., 2018). In addition, Serda et al. also evaluated structure-activity relationship of several TSCs containing retro fragments that have been used in previously designed TSCs. They have demonstrated that use of an N4-based piperazine or morpholine ring in TSCs resulted in more potent and selective anti-cancer activity in various cancerous cell lines (Serda et al., 2014). In another study, Serda et al. have also showed that quinoline-based thiosemicarbazones have greater anti-proliferative activity than halogenophenyl compounds (Serda et al., 2012). In line with these previous observations, compound 17b showed promising anti-cancer activities with potent and selective anti-proliferative and apoptotic activity, which further proposed that the exchange of the piperidine group including the S form of the chiral thiosemicarbazone improved anticancer activity cancer cell lines (Tasdemir et al., 2015).

CONCLUSIONS

Here we demonstrated an as yet undetermined anti-cancer property of a novel thiosemicarbazone derivative 17B which induce cell cycle arrest and trigger apoptosis by modulating cellular signaling pathways and reduce the migration capacity of cancer cells. Although there are several limitations such as the lack of in vivo effects of 17B, findings of the present have strongly indicated that 17B might be a novel anti-cancer drug for the treatment of breast cancer and osteosarcoma but not for lung cancer. However, there are some limitations of our study,

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FIGURE LEGENDS

Figure 1. Chemical structure of 17B ((S)-(+)-2-[(4-piperidin-1-il)benzilidin])-N-(1-feniletil)hidrazinkarbotiyoamid).

Figure 2. 17B restricts cell viability and exerts cytotoxicity in a dose-dependent manner. Cells were treated with either with controls or 17B and analyzed for cell viability, LDH activity and morphology. 17b significantly impaired with the viability of cancer cells and induced cytotoxicity in all cancer cells. Also, significant morphological changes were observed.

Figure 3. 17B induces apoptosis and activates caspase-mediated killing. Cells were treated with either with controls or 17B and cell cycle were analyzed to observe induction of apoptosis and activation of pro-apoptotic members as well as inactivation of anti-apoptotic members. 17B strongly induced apoptosis of MCF7 and U2OS cells but not A549 cells and modulated expression levels of pro-apoptotic and anti-apoptotic mediators in MCF7 and U2OS cells.

Figure 4. 17B impairs progression through cell cycle and modulates regulators of cell cycle. Cells were treated with either with controls or 17B and cell cycle were analyzed to observe cell cycle arrest. 17B significantly induced cell cycle arrest in in MCF7 and U2OS cells by interfering with the G1/S transition and modulating genes responsible from the regulation of cell cycle progression.

Figure 5. 17B interferes with the migration of cancer cells. Cells were treated with either with controls or 17B and wound closure were observed in a time-dependent manner. 17B interfered with the migration of cancer cells and significantly delayed wound healing. The black arrows were used to indicate the boundary lines of scratch.

Figure 6. Mechanism of action of tumor suppression mediated by novel thiosemicarbazone derivative 17B. 17B impairs progression through cell cycle by interfering with the G1/S transition by modulating genes involved in the regulation of cell cycle. Also, it triggers apoptosis by activating the expression levels of pro-apoptotic (BAX, CASP3 and CASP9) and inhibiting the expression of anti-apoptotic mediators (BCL2 and BCLxl) and reduces the migration capacity of cancer cells.

ATCC	American Type Culture Collection
DMSO	Dimethyl-Sulfoxide
FCS	Fetal Calf Serum
FACS	Fluorescence-Activated Cell Sorting
HBSS	Hank's Balanced Salt Solution
LDH	Lactate Dehydrogenase
PI	Propidium Iodide
TSCs	Thiosemicarbazones

Authors Contributions: E.B. designed the study and performed experiments. D.T.K. and A.K.I. designed and synthesized thiosemicarbazone derivative 17B and performed its chemical characterization. K.A and I.B helped for cell culture experiments. E.B. and I.B. analyzed the data, performed statistics and drafted manuscript. E.A.C. supervised this project and edited final draft.

Highlights

- 17B restricts cell viability and exerts cytotoxicity in a dose-dependent manner
- 17B induces apoptosis and activates caspase-mediated killing
- 17B inhibits progression through cell cycle by interfering G1/S transition
- 17B interferes with the migration of cancer cells