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Synthesis And In Vitro Evaluation Of Cytotoxic Effect On Cervical Cancer Cells (HELA) Of Tetrahydroquinoline-Isoxazole Hybrid Derivatives.

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ABSTRACT

Tetrahydroquinolines and isoxazoles are heterocyclic compounds extracted from natural sources that have shown a wide range of pharmacological properties, including cytotoxic and antitumor properties. Based on the above, it is expected that hybrids of these two molecules potentiate their action and have greater anticancer effects.Cytotoxic activity of tetrahydroquinoline and isoxazole hybrid compounds on cervical cancer cells (HeLa) was evaluated. Cytotoxic effect of eight hybrid compounds was carried out by the MTT method. For the compound with the highest cytotoxic activity, morphological analysis was performed and cytochrome crelease, effect on mitochondrial permeability transition pore (mPTP) and DNA fragmentation were determined.Fourcompounds showed cytotoxic concentrations (CC_{50}) less than 100 µM after 48 hours of treatment.Hybrid compound **4b**showed thehighestcytotoxic effect with a $CC_{50}$of 19.7 ± 2.7 µM at 72h. In addition, the effect of this hybrid compound increased three times the of cytochrome c releasewith respect to control, inhibited the mPTP and induced DNA fragmentation. Tetrahydroquinoline and isoxazolehybrid compounds showed a cytotoxic effect on HeLa cervical cancer cells and the hybrid compound **4b** inducedcharacteristic alterations of mitochondrial apoptosis in these tumor cells.

Keywords: Cancer, tetrahydroquinolines, isoxazoles, cytotoxicity, apoptosis, hybrids.

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INTRODUCTION

Cancer is a series of diseases that are produced by the uncontrolled proliferation of cells that have evaded different cell cycle controls[1]. Annually around 8.2 million people worldwide die of cancer, of which approximately 266,000 deaths are caused by cervical cancer. In Colombia, this specific type of cancer represents a public health problem being the second cause of cancer death in women [2,3].

For this reason, different methods have been used to treat this disease; among which are radiotherapy, surgery and chemotherapy. However, the effectiveness of availabledrugs in cancer chemotherapyusually varies with respect to the neoplasia stage and the state of the patient and the treatment can also cause differentside effects such as alopecia and nausea[4].All these factors have promote the continuous search for new medications and its exploration has been directed to natural sources for the search of potential drugs.

Natural products have played a key role in the identification of new bioactive molecules, including compounds with antibacterial, antifungal, antituberculosis and antitumor activities. However, the process of discovering drugs from natural sources is a difficult, tedious and time-consuming process. Currently, drug discovery applies tools of synthetic chemistry as an innovative approach aimed at exploringand identifyingbioactive molecules thatincrease the chances of becoming novel drugs.

By organic synthesis is possible to generate copies of molecular structures of interest and develop hybrid compounds, which allow to put pharmacophores in the correct position to obtain beneficial effects in their clinical application [5–9]. In this sense, tetrahydroquinolines and isoxazoles are compounds that arouse interest for their anticancer activity demonstrated in different investigations [10–16].

Tetrahydroquinolines are aromatic heterocyclic molecules derived from quinolines that have different biological properties such as antimalarial, antiasthmatic and anticancer activities[17]. Within tetrahydroquinoline compounds with anticancer activity can be foundthe 2-amino-3-cyano-8-methyl-4-substituted-5,6,7,8-tetrahydroquinoline derivatives with activity on Ehrlich-Lettreascites carcinoma cells (EAC) andwith the capacity of inhibit the growth lung, leukemia, melanoma, breast, renal and prostate cancer cell lines in percentages higher than 60% [13].Fused tetrahydroquinolines such as the compound N'-(3-cyano-4,7,7-trimethyl-5-oxo-5,6,7,8-tetrahydroquinolin-2-yl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)formimidamide have showedpotent cytotoxic activityagainst breast cancer (MCF7)[14].

On the other hand, isoxazoles are heterocyclic compounds containing two heteroatoms, nitrogen and oxygen, thesecompounds have also shown antibacterial, anti-tuberculous and anti-carcinogenic therapeutic properties [18]. For example, the series of isoxazoles obtained from phenylbenzothiazole derivatives, demonstrated cytotoxic activity in three cell lines, lung adenocarcinoma, colon cancer and breast cancer [19]. Similarly, the compound 3,3'-isoxazole-3,5-diyl-bis(5-methoxy-1-methyl-1H-indole) resulted active in different lines of human carcinoma [12], while cis-fused isoxazoles with chromene possess anticancer activity against four cell lines (MDA-MB-231, MCF-7, A549 and HeLa) [16].

In the present investigation the cytotoxic activity of eighthybrid compounds (tetrahydroquinoline-isoxazole) were tested on human cervical adenocarcinoma cells (HeLa). In addition, the ability to release cytochrome c and to fragment DNA, two phenomena that occur during cell death by apoptosis,were established [20,21].

EXPERIMENTAL SECTION

Chemistry

All reagents were purchased from Sigma and Aldrich Chemical Co., Merck and J.T. Baker and were used without further purification. Aqueous solutions were prepared using Milli Q water. Reaction progress was monitored using thin layer chromatography on PF254 TLC aluminum sheets from Merck. Column chromatography was performed using Silica gel (60 – 120 mesh) and analytical gradesolvents were employed. The melting points (uncorrected) were determined using a Fisher-Johns melting point apparatus. IR spectra were recorded on a FT-IR Bruker Tensor 27 spectrophotometer coupled to Bruker platinum ATR cell. Mass

spectrometry ESI-MS analyses were conducted on an ESI-IT Amazon X (Bruker Daltonics) with direct injection. NMR spectra (^{1}H and ^{13}C) were measured on a Bruker Ultrashield-400 spectrometer (400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR), using $CDCl_3$ as solvent and TMS as reference. J values are reported in Hz; chemical shifts are reported in ppm (δ) relative to the solvent peak (residual $CHCl_3$ in $CDCl_3$ at 7.26 ppm for protons). Signals were designated as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; t, triplet; td, triplet of doublets; q, quartet; m, multiplet and br., broad.

General procedure for the synthesis of molecular hybrid Tetrahydroquinoline/Isoxazole 4a-h.

The first synthetic step for obtaining the tetrahydroquinoline/isoxazole hybrids of interest consisted in the preparation of the corresponding N-propargyl tetrahydroquinolines **2a-d** via three-component cationic Povarov reaction[22]. A solution of the corresponding N-propargyl-4-(2'-oxopirrolidin-1'-il)-1,2,3,4-tetrahydroquinolines (1 mmol) and the respective aldoximes **3a-b** (3 mmol) in dichloromethane (10 mL), was poured into a 50-mL roundbottom flask and vigorously stirred at room temperature for 10 min. Then, triethylamine (TEA) (0.1 mmol) was added and the reaction was placed in ice bath until the temperature dropped to 0 °C, subsequently 10 mL of solution 10% w/v of NaOCl (8.6 mmol) was added drop by drop to the mixture. The reaction was monitored by TLC and after 4–6 hours, the mixture was diluted with water (30 mL) and extracted with ethyl acetate (3 x 20 mL). The organic phase was separated and dried over Na_2SO_4, concentrated in vacuo and the resulting products**4a-h** were purified bycolumn chromatography (silica gel, petroleum ether-ethyl acetate).

Selected spectral data: 5-((4'-(2''-Oxopyrrolidin-1''-yl)-3',4'-dihydroquinolin-1'(2'H)-yl)methyl)-3-phenylisoxazole **4a**
IR (ATR): 3465.2, 2931.2, 1670.0, 1497.2, 1438.9, 1167.6, 1017.5, 665.2 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.95-2.05 (2H, m, 4''-H), 2.05-2.23 (2H, m, 3'-H), 2.47-2.51 (2H, m, 3''-H), 3.10-3.27 (2H, m, 5''-H), 3.39-3.60 (2H, m, 2'-H), 4.56 (1H, d, J = 17.5 Hz, 11'-Ha), 4.62 (1H, d, J = 17.5 Hz, 11'-Hb), 5.41 (1H, dd, J = 9.2, 5.4 Hz, 4'-H), 6.37 (1H, s, 4-H), 6.66 (1H, d, J = 8.6 Hz, 8'-H), 6.70 (1H, dd, J = 7.4, 1.0 Hz, 5'-H), 6.89 (1H, td, J = 7.4, 1.1 Hz, 6'-H), 7.10 (1H, tdd, J = 7.8, 1.6, 0.7 Hz, 7'-H), 7.40-7.43 (3H, m, 3-H_{Ar}, 5-H_{Ar}, 4-H_{Ar}), 7.72-7.76 (2H, m, 2-H_{Ar}, 6-H_{Ar}); **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 18.4, 26.7, 31.5, 43.8, 47.7, 48.0, 48.2, 100.4, 112.0, 117.1, 120.1, 126.9, 127.9, 128.8, 128.8, 129.0, 129.0, 130.2, 145.0, 162.5, 170.0, 175.7; **MS** (ESI-IT) m/z: 289.1 [M-C_4H_8NO]$^+$, 374.1 [M+H]$^+$, 396.1 [M+Na]$^+$. 5-((4'-(2''-Oxopyrrolidin-1''-yl)-3',4'-dihydroquinolin-1'(2'H)-yl)methyl)-3-(3,4,5-trimethoxyphenyl)isoxazole **4b IR** (ATR): 3437.0, 2939.4, 1668, 1421.5, 1236.3, 11245.6, 1001.0. 842.9 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.89-2.25 (4H, m, 4''-H, 3'-H), 2.44-2.56 (2H, m, 3''-H), 3.12-3.35 (2H, m, 5''-H), 3.36-3.70 (2H, m, 2'-H), 3.87 (3H, s, 4-OCH_3), 3.91 (6H, s, 3-OCH_3, 5-OCH_3), 4.51 (1H, d, J = 16.7 Hz, 11'-Ha), 4.64 (1H, d, J = 16.7 Hz, 11'-Hb), 5.34-5.47 (1H, m, 4'-H), 6.32 (1H, s, 4-H), 6.56 (1H, d, J = 7.1 Hz, 5'-H), 6.64 (1H, d, J = 10.1 Hz, 8'-H), 6.81-6.85 (1H, m, 7'-H), 6.92-7.06 (2H, m, 2-H_{Ar}, 6-H_{Ar}), 7.27-7.31 (1H, m, 6'-H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 18.3, 26.4, 31.2, 43.5, 47.8, 48.4, 50.0, 56.4, 56.4, 61.0, 100.5, 104.0, 104.0, 113.2, 122.0, 122.5, 124.1, 127.1, 128.6, 139.6, 143.5, 153.7, 153,7, 162.4, 169.5, 175.0; **MS** (ESI-IT) m/z: 486.0 [M+Na]$^+$. 5-((6'-Methyl-4'-(2''-oxopyrrolidine-1''-yl)-3',4'-dihydroquinolin-1'(2'H)-yl)methyl)-3-phenylisoxazole **4c**

IR (ATR): 3374.9, 2952.5, 1667.7, 1421.3, 1285.8, 1093.9, 905.4. 694.7 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.95-2.10 (2H, m, 4''-H), 2.15-2.24 (2H, m ,3'-H), 2.19 (3H, s, 6'-CH_3), 2.48-2.52 (2H, m, 3''-H), 3.10-3.27 (2H, m, 5''-H), 3.36-3.56 (2H, m, 2'-H), 4.54 (1H, d, J = 17.4 Hz, 11'-Ha), 4.59 (1H, d, J = 17.4 Hz, 11'-Hb), 5.38 (1H, dd, J = 9.0, 5.4 Hz, 4'-H), 6.36 (1H, s, 4-H), 6.58 (1H, d, J = 8.4 Hz, 8'-H), 6.71 (1H, d, J = 1.8 Hz, 5'-H), 6.91 (1H, dd, J = 8.4, 2.1 Hz, 7'-H), 7.40-7.43 (3H, m, 3-H_{Ar}, 5-H_{Ar}, 4-H_{Ar}), 7.72-7.76 (2H, m, 2-H_{Ar}, 6-H_{Ar}); **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 18.4, 20.5, 27.0, 31.6 , 43.9, 47.9, 47.8, 48.2, 100.4, 112.2, 120.2, 126.9, 126.9, 127.0, 128.5, 128.9, 129.0, 129.0, 129.4, 130.1, 142.8, 162.5, 170.2, 175.7; **MS** (ESI-IT) m/z: 410.1 [M+Na]$^+$, 797.2 [2M+Na]$^+$.5-((6'-Methyl-4'-(2''-oxopyrrolidin-1''-yl)-3',4'-dihydroquinolin-1'(2'H)-yl)methyl)-3-(3,4,5-trimethoxyphenyl)isoxazole **4d**

IR (ATR): 3392.7, 2935.6, 1668.4, 1421.5, 1124.5, 1001.0, 524.9 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 1.95-2.05 (2H, m, 4''-H), 2.06-2.23 (2H, m, 3'-H), 2.19 (3H, s, 6'-CH_3), 2.47-2.55 (2H, m, 3''-H), 3.12-3.28 (2H, m, 5''-H), 3.37-3.58 (2H, m, 2'-H), 3.86 (3H, s, 4-OCH_3), 3.90 (6H, s, 3-OCH_3, 5-OCH_3), 4.51 (1H, d, J = 17.6 Hz, 11'-Ha), 4.62 (1H, d, J = 17.6 Hz, 11'-Hb), 5.4 (1H, dd, J = 9.2, 5.6 Hz, 4'-H), 6.32 (1H, s, 4H), 6.56 (1H, d, J = 8.4 Hz, 8'-H), 6.7 (1H, d, J = 2.0 Hz, 5'-H), 6.91 (1H, dd, J = 8.4, 2.0 Hz, 7'-H), 6.96 (2H, s, 2-H_{Ar}, 6-H_{Ar}); **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 18.4, 20.5, 26.9, 31.6, 43.8, 48.0, 48.0, 48.4, 56.4, 56.4, 61.1, 100.3, 104.1, 104.1,

112.2, 120.2, 124.6, 128.3, 129.4, 133.6, 139.6, 142.8, 153.6, 153.6, 162.4, 170.3, 175.7; **MS** (ESI-IT) m/z: 478.2 $[M+H]^+$, 500.2 $[M+Na]^+$.

BIOLOGICAL ACTIVITY

Reagents

EMEM, trypsin, MTT, TRIS-HCl, digitonin, Wright Giemsa stain and SYBR greenwere purchased from Sigma (St. Louis, MO, USA). Analytical grade reagents were used and solutions were prepared in Milli Q water.

Animals

Male Wistar rats (~180-200 g) were supplied by the Universidad Industrial de Santanderbioterium. Individuals were maintained in a temperature-controlled room (22 ± 1ºC) with free access to food and water under 12 h light/12 h dark cycle. These animals were starved for 12 h and then killed by decapitation. All procedures were conducted following the recommendations of Colombian Law 84 of 1989 (Chapter IV, articles 23-26) and the Resolution 8430 of 1993 (Title IV, articles 83-93) for the scientific management of animals and procedures and were approved by the Institutional Ethics Committee.

Cell culture

Cervical cancer cells (HeLa) were cultured in EMEM medium (Eagle's Minimum Essential Medium) supplemented with 10% Fetal Bovine Serum (FBS) and 50 µg / mL of gentamicin. Cells were maintained at 37 ° C and 5% CO_2 ina humidified atmosphere. The cultures at ~ 80% confluence were sub-culturing in flasks of 25 mL by addition of solution of trypsin-EDTA.

Cell viability

MTT assay

The effect of the hybrid THQ-isoxazole compounds on the viability of HeLa cells was carried out by the MTT method. HeLa cells were deposited ($1x10^4$ cells / well) in 96-well plate and incubated for 24 hours at 37 °C with a 5% CO_2 stream. Next, cells were treated with differentconcentrations of the hybrid compounds (5, 25, 50 and 100 µM) for 48 hours. Subsequently, culture medium was replaced by 200 µL of MTT (0.5 mg / mL in HBSS) and kept in incubation for 3 hours at 37 ° C. Finally, the formazan crystals were dissolved in dimethylsulfoxide (DMSO) and the absorbance was measured at 580 nm [23]. With the most cytotoxic compound, the effect on cell viability was analyzed in different treatment times (12, 24, 28 and 72 hours).

Crystal violet assay

The effect of the hybrids, in low concentration, on the viability of HeLa cells was carried out by the crystal violet method. HeLa cells were deposited ($5x10^3$ cells / well) in 96-well and incubated for 24 hours at 37°C with a 5% CO_2 stream. Next, cells were treated with two concentrations (5 y 25) µM of the most cytotoxic compound for 48 hours. Subsequently, cells were washed with phosphate buffer saline(PBS: containing 680.0 mM NaCl, 13.4 mM KCl40.5 mM Na_2HPO_4), and fixed with methanol for 10 minutes. Thencells were incubated with 50 µL of (0.02% p/v) crystal violet for 5 minutes. Finally, cells were washed 10 times with PBS and the crystals were solubilized with 200 µL of (0.05 M) sodium citrate. The absorbance was measured at 550 nm [24].

Morphological analysis

HeLa cells ($1x10^5$ cells / well) were seeded in slides in 12-well plates and incubated with 5% CO_2 atmosphere at 37 ° C for 24 h. After adhesion, cells were treated with the IC_{50} of the most cytotoxic compound for 24 and 48 h. After the treatment time, cells were stained with wright giemsa, following the manufacturer's protocol. The morphological changes were examined with a Leica microscope, $ICC_{50}W$ camera.

Cytochrome C release

Cells ($1x10^6$) were incubated in Petri dishes (60 mm diameter) for 24 hours. Subsequently, they were treated with the highest cytotoxicity compound with a 25µM concentration for 12 hours. After the treatment time, cells were released with trypsin-EDTA, centrifuged and washed with PBS, maintaining a volume of 1mL. An aliquot of 30 µL were separated for protein quantification [25]. The remaining suspension was centrifuged at 4500 X g for 15 minutes. Subsequently, pellet was resuspended in 1 mL of 85.55 mM TRIS-HCl pH 7.2, 7.89 mM EDTA, 0.146 mM sucrose and 10 µM digitonin, followed by incubation for 30 min at 4 ° C. Then, the cell suspension was centrifuged at 10,000 X g for a period of 30 minutes, the supernatant was filtered with the help of a polystyrene membrane with a pore diameter of 200 nm and its absorbance was quantified at 414 nm. Finally, the concentration of cytochrome c released was calculated [26].

Mitochondrial permeability transition

Mitochondria were isolated from rat liver by differential centrifugation according to the protocol described by Voss et al.[27], using an extraction medium consisting of 250 mM D-mannitol, 10 mM HEPES, 1 mM EGTA and BSA 0.1% w/v (pH 7.4) at 4ºC and the same medium without BSA to suspend the intact mitochondria isolated. Mitochondrial preparations with a respiratory control above 3.0 were used. Mitochondrial permeability transition experiments were assayed spectrophotometrically according to Bernardi [28]. Standard reaction medium contained mitocondrial suspension (0.5mg protein), 250 mM D-mannitol, 10 mM HEPES buffer (pH 7,2), 4 mM potassium glutamate and 45 µM de $CaCl_2$. Swelling for opening the pore was induced by the addition of 0.3 mM potassium phosphate and 10 µM H_2O_2. The swelling by opening the pore was confirmed by individual addition of 1.0 mM dithiothreitol (DTT), 1.0 mM EGTA and 0.5 mM cyclosporine A (CsA).

DNA fragmentation

Cells ($5x10^5$) were seeded in 60 mm Petri dishes until complete adhesion. The plates were then treated with the compound that showed the higher cytotoxic effect at a concentration of 25 µM for 24 and 48 hours. After treatment, the cells were washed with PBS, scraped and the DNA was extracted using anApoTarget DNA ladder detection kit from Invitrogen Corporation (Camarillo, CA, USA), according to the manufacturer's instructions. Finally, the DNA samples were separated electrophoretically on a 1% agarose gel at 300 mA, 12 V / cm for 40 minutes at 25 ° C. The gel was stained using SYBR green and the images were acquired using a transilluminator with a BioDoc-it UVP image capture. Treatment with 10 µM camptothecin for 5 hours at 37 ° C was used as a positive control.Quantity marker 1kb of Gene Mate was used as a molecular weight marker.

Protein determination

Protein concentrations were determined by the Bradford et al.method [25], using BSA as standard.

Statistical analysis

Data are presented as mean ± S. D (standard deviation). The statistical analysis was performed by of variance (ANOVA) andfollowed by Tuckey's test for averages comparison. The results were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Hybrid compounds are an alternative in the development of new drugs that allow greater beneficial effects. In this sense, tetrahydroquinoline and isoxazolederivatives present a wide range of biological properties, including anti-inflammatory, anti-arrhythmic, immune suppressors, antiviral, antituberculosis, antioxidants [17,29], antifungal, antibacterial, anticonvulsant, antidepressant and anticancer activities [30-32]. Therefore, in the present study, the analysis of the cytotoxic activity of eight hybrid compounds (tetrahydroquinoline and isoxazole) on HeLa cells was performed. In addition, biochemical characteristics of the type of death that triggers the cytotoxic effect were analyzed.

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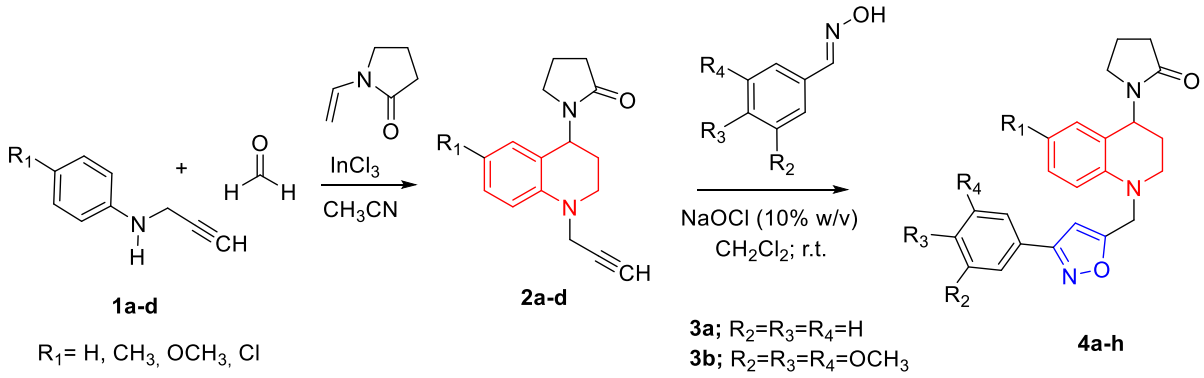
Chemistry results

Tetrahydroquinoline-isoxazole hybrids

The eight hybrid compounds analyzed in this work are formed by the tetrahydroquinoline and isoxazole heterocycles. For access to the hybrids compounds of interest, the respective N-propargyl tetrahydroquinolines **2a-d** were synthetize. Thesecompounds were obtained via one-pot cationic Povarov reaction, and then was transform to the corresponding hybrids **4a-h**, through the 1,3-dipolar cycloaddition reaction (Scheme 1). All compounds **4a-h** were obtained as reddish viscous oils with good yields reaction (42-85%) after column chromatography purifications. ^{1}H-NMR and ^{13}C-NMR analysis allowed to carry out the complete structure characterization of the molecular hybrids **4a-h**. It is worth noting that isoxazole fragment present in the molecular hybrids has a phenyl and trimethoxyphenyl group. The difference of the nature of these substituents makes it possible to determine whether the presence of the trimethoxyphenyl group significantly enhances the cytotoxic activity of the hybrid compounds.



Scheme 1: Synthetic scheme for access to hybrids of tetrahydroquinoline and isoxazoles.

Biological results

Tetrahydroquinoline- isozaxolehybrid compounds affect the viability of HeLa cells.

Tetrahydroquinoline-isoxazole hybrid compounds were evaluated by the MTT method for 48 hours, using concentrations of 5, 25, 50 and 100 µM.Cell viability is presented as the cytotoxic concentration 50 (CC_{50}). Among the tested compounds, fourresulted possess cytotoxic effect on HeLa cells, with CC_{50} values less than 100 µM (Table 1).

The compounds evaluated that showed the highest cytotoxicity in decreasing order were **4b**>**4e**>**4g**>**4d**. From these results not was possible to establish a relationship between the structure of the derivatives and the cytotoxic effect shown. However, it was observed that when the trimethoxyphenyl group in the isoxazole fragmentis present, the presence of substituentsdifferent to proton in C-6 position of the tetrahydroquinoline leads to a marked decrease in the cytotoxic effect. In the homologous compounds **4b** and **4d**, the change from proton (compound **4b**) to the methyl (compound **4d**)generated an increase in the CC_{50} from 34.5 to 63.9 µM. In the case of hybrids with a phenyl group in the isoxazole ring, the viability of HeLa cells increased with the electronegativity of the substituent in position C-6 of the tetrahydroquinoline fragment, e.g methoxy group (**4e**, CC_{50}= 52,9 µM) showeda higher cytotoxic activity than the chlorine atom (**4g**,CC_{50}= 58,1µM).

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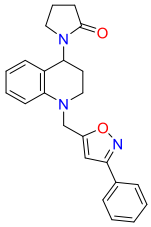
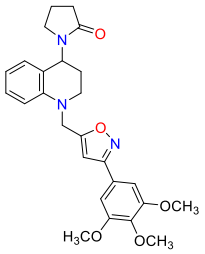
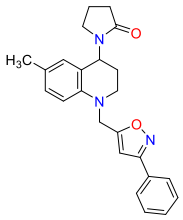
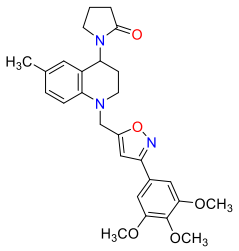
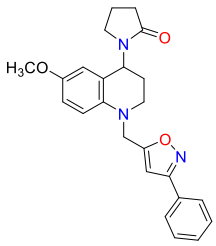
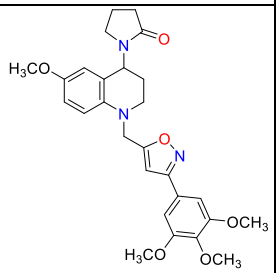
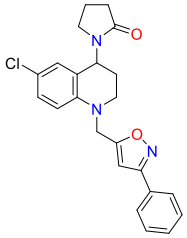
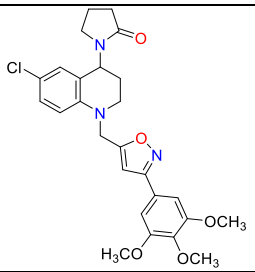
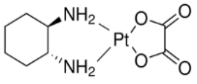
Table 1: Cytotoxic activity of new tetrahydroquinoline/isoxazole hybrids on HeLa cells4a-husing MTT assay.

Comp.	Structure	CC_{50}[a][µM]	Comp.	Structure	CC_{50}[a][µM]
4a		Nd	**4b**		34.5 ± 10.3
4c		>100	**4d**		63.9 ± 1.9
4e		52.9 ± 13.4	**4f**		>100
4g		58.1 ± 8.2	**4h**		>100
OHP [b]		**14.3 ± 2.8**			

[a] CC_{50} value corresponding to the compound concentration required to inhibit cellular viability by 50%. [b] Oxaliplatin (OHP) was used as positive control. Nd: not determinate. Data represent three independent experiments, each in triplicate.

Among the compounds that exhibit cytotoxic activity, compound **4b** was highlighted with the lowest CC_{50}value (34.5 ± 10.3), so additional tests were carried out. To analyze the effect of the hybrid **4b** over time, cytotoxic capacity was evaluated by 12, 24, 48 and 72 hours of treatment, using concentrations of 5, 25, 50 and 100 µM (Figure 2). The obtained results showed that compound **4b** had a dose-dependent and time-dependent effect. In a period of 12 h and 24 h, 50 and 25 µMconcentrations generated a ~ 20% reduction in cell viability. Also, a low concentration (25 µM), after 48 and 72 h, was able to decrease cell viability by ~ 38 and 45%, respectively, and a high concentration (100 µM) after 48 h of treatment generated ~ 80% reduction in the viability of HeLa cells.

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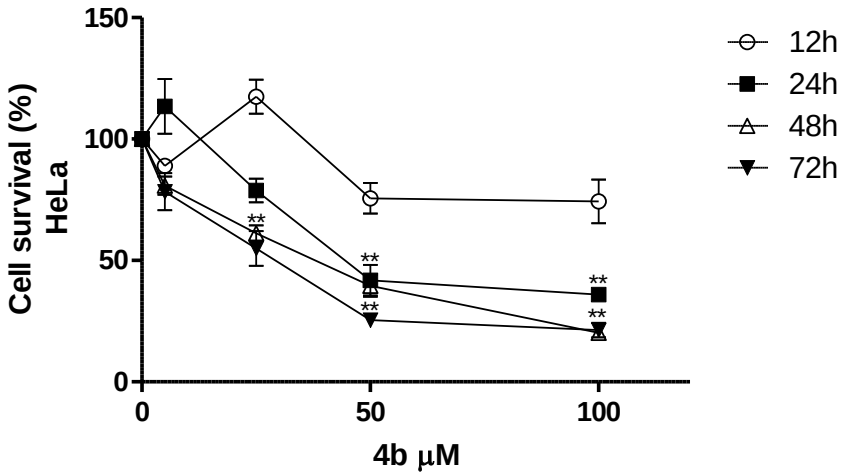

Figure 2: Cytotoxic effect of hybrid 4b on HeLa cells for 12, 24, 48 y 72 h measured by the MTT assay. Values represent the mean ± SD of percentage of viable cells in comparison to the control (data represent three independent experiments, each in triplicate). ** Statistically significant differences compared to the control p<0.01.

The CC_{50} of compound **4b** at different times confirms the dependence of the effect with respect to the treatment time. Although the CC_{50} at 12 h is evidently high, it is possible to observe the gradual decrease in this concentration at each time evaluated (Table 2).Additionally, compound **4b** was evaluated by the crystal violet method for 48 hours of treatment, using low concentrations (5 and 25 µM) and the results showed that the hybrid**4b** decreased the cell viability in 39.1 and 69.5 %, respectively (Figure 3). These results are consistent with those reported by other authors, where the presence of a trimethoxyphenyl group in polyfunctionalized molecules and diverse chemical structures, isolated of natural products, possess potent anticancer properties [34].

Table 2:Values of CC_{50} of hybrid 4b with respect to time

Treatment time (hours)	CC_{50} µM*
12	>100
24	51, 1 ± 4,9
48	34,5 ± 10,3
72	19,7 ± 2,7

*CC50 value corresponding to the compound concentration required to inhibit cellular viability by 50%. Data represent three independent experiments, each in triplicate.

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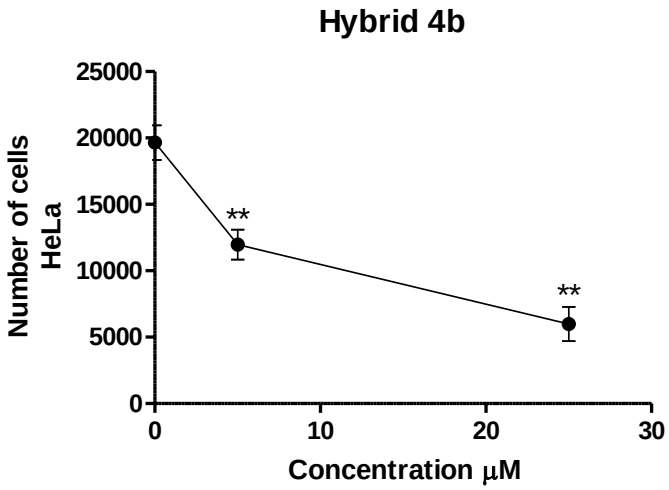

Figure 3: Cytotoxic effect of 4b hybrid on Hela cells for 48h measured by crystal violet assay. Values represent the mean ± SD of percentage of viable cells in comparison to the control (data represent three independent experiments, each in triplicate). ** Statistically significant differences compared to the control p<0.01.

It is important to note that all the compounds evaluated are present as a racemic mixture of two stereoisomers(50:50), since they have a chiral carbon in their structure. In the literature, several examples of compounds with spatial isomers are reported, of which only one possesses the biological activity [35-37]. This enables only one of the isomers of compound **4b** to present the cytotoxic effects reported here and, therefore, its CC_{50} may be lower.

Hybrid compound 4b alters the morphology of HeLa cells and inducesthe cytochrome crelease

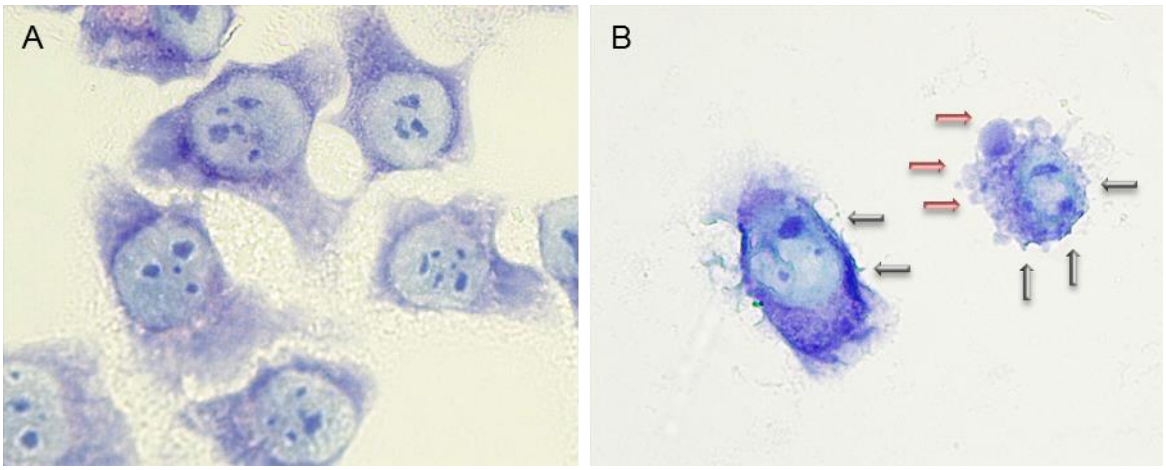

Figure 4: Effect of compound 4b on the morphology of HeLa cells. The cells were incubated for 48 hours at 25 µM. The images were taken with a Leica microscope, 40x objective. Cells without treatment (a). Treatment with 4b to 25 µM (b).

In order to explain the cytotoxic effect presented by**4b** hybrid and to know the mechanism of cell death, different methods, based on morphological and biochemical characteristics, were used. Morphological analysis of compound **4b** was performed at 25 µM for 48 hours of treatment. While for HeLa cells without treatment a typical adherent morphology with monolayer formationwasobserved (Figure 4a), in the cells treated with the hybrid **4b** there was a contact loss with adjacent cells, reduction of cell volume and appearance of blebs in the plasma membrane (whichsuggest their destabilization and the presence of apoptotic bodies)(Figure 4b). Additionally, cells treated for 12 hours at a concentration of 25 µM showed 100%

of cell viability, so it would be expected to find initial phases of induction of cell death. Compound **4b** induced, in approximately380%, the release of cytochrome c to the cytoplasm giving clear indications of a possible induction of programmed cell death (Figure 5)[38, 39].

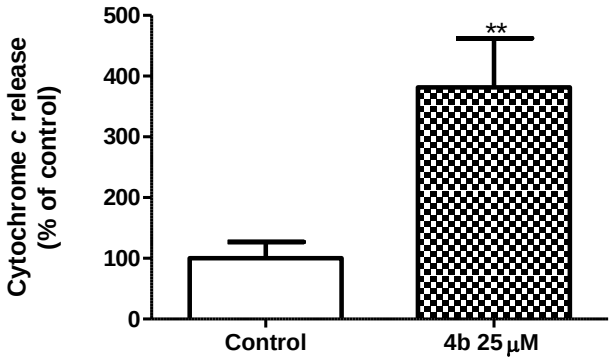

Figure 5: Effect of hybrid 4b on the cytochrome c released. Values represent the percentage of the control (100%). The control is 0.52 nmol de cytochrome c released min-1 mg-1 of total protein. Data represent three independent experiments, each in triplicate. ** Statistically significant differences compared to the control p<0.01.

Because the treatment with compound **4b**generated an evident cytochrome c release, this result suggested that this hybrid could activate the apoptotic process by an intrinsic pathway. The control and regulation of these apoptotic mitochondrial events occurs through of the Bcl-2 family of proteins. These proteins have special significance since they can regulate of cytochrome c release and the activity of proteins involved in pro-apoptosis. The extrinsic pathway ends at the execution phase. Caspases 3, 6 and 7 functionsas executioner caspases, cleaving various substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells begins this phase of apoptosis[40, 41]. Likewise, the morphological analysis showed characteristics of cell death due to apoptosis, cellular contraction, loss of contact with the matrix and formation of grooves in the membrane (Figure 4b). In this sense, it was reported that these morphological characteristics are the result of the action of caspases that cloned many of the constituents of the cytoskeleton, allowing cellular separation and groove formation in the membrane and generating blebs in the weakened cytoskeleton [42,43].

Hybrid compound 4b inhibits the MPTP and induces DNA fragmentation in HeLa cells

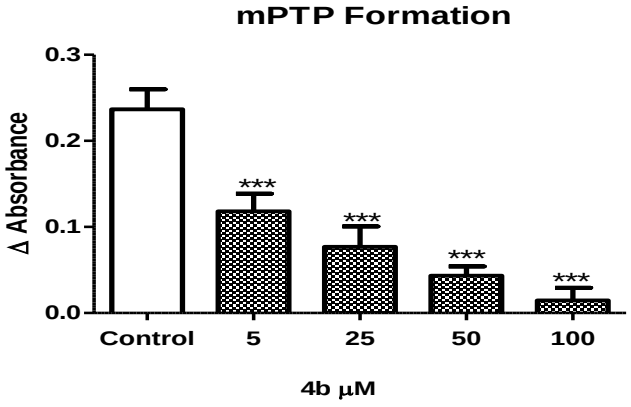

Figure 6: Effect of hybrid 4b on the mitochondrial permeability transition pore (mPTP) in mitochondria isolated from rat liver. Data represent three independent experiments, each in triplicate. ***Statistically significant differences compared to the control p<0.001.

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To better understand the mechanism of cell death induced by the **4b** hybrid, the effect of this compound was evaluated in mitochondrial permeability transition pore (mPTP) experiments on mitochondria isolated from rat liver. Results showed that while a concentration of 5 µM was able to close the pore by ~ 50%, a high concentration of 100 µM did it by ~ 90%(Figura 6). Several studies indicate that sustained opening of mPTP is most likely involved in necrotic cell death rather than apoptosis or autophagy [44, 45]. Also, there are reports of other compounds that induce apoptosis via mitochondrial pathway in human cells, independent of the mitochondrial permeability transition pore [46]. Likewise, many lines of evidence support the notion that the mitochondrial apoptosis-induced channel (MAC) directly and selectively induces the mitochondrial outer membrane permeabilization (MOMP), without disrupting of the integrity of the mitochondrial inner membrane, to release cytochrome c and other pro-apoptotic factors from the intermembrane space [44, 47].This may be consistent with the fact that caspases operate in an ATP-dependent manner and recent evidence demonstrates the role of ATP synthase in the mPTP formation [48-50]. Thus, mPTP may drive necrosis or regulated apoptosis depending on the intracellular ATP availability [48].

On the other hand, DNA fragmentation capacity was analyzed as another characteristic of programmed cell death [51, 52]. DNA extraction of HeLa cells treated with hybrid **4b** was carried out at a concentration of 25 µMfor 24 and 48 hours (lanes 2 and 3, Figure 7) and the results showed a DNA separation pattern in fragments multiples of 300bp. When DNA of the HeLa cells without treatment was analyzed a single band corresponding to integrated DNA was presented(lane 1, figure 5). As positive control, DNA from cells treated with 10 µM camptothecin for 5 hours was isolated(lane 4, Figure 5). The literature reports the use of camptothecin as an inducer of apoptosis with induction of DNA fragmentation[53],however a more defined pattern was evidenced with the treatment of compound **4b** than with camptothecin.This is a typical feature of the mechanism of cell death, it is considered a key event in the development of apoptosis and a strong indicator of the presence of this process [42].

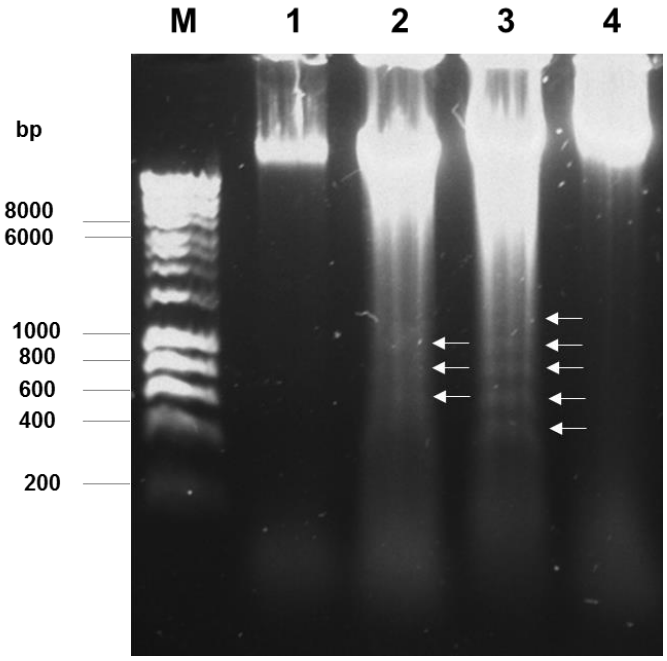

Figure 7: Agarose gel electrophoresis of DNA extracted from HeLa cells exposed to hybrid 4b (25 µM) for 24 and 48 h. M) size marker. 1) Control cells without compound. 2) Cells treated with hybrid 4b for 24 h. 3) Cells treated with hybrid 4b for 48 h. 4) Cells treated with Camptothecin(CPT) 10 µM for 5 h.

These results suggested that at least oneof the tetrahydroquinoline - isoxazole hybrids has the abilityto induce apoptosis via mitochondrial pathway in human cervical cancer cells independent of the mPTP and is a promising compound to continue with the separation of its isomers and studies of its mechanism of action.Obtained results supported further investigations with new hybrid compounds of the series evaluated here and they are now being synthesized and evaluated on different cell lines in our laboratories and their results will be published elsewhere.

CONCLUSION

Tetrahydroquinolines and isoxazolehybrid compounds presented a cytotoxic effect on HeLa cells, where hybrid **4b**had the highest cytotoxic effect of the series evaluated. Hybrid **4b** affected the viability and proliferation of cells and generated changes in cell morphology, cytochrome c release, inhibition of the mPTP, and DNA fragmentation. This suggested that the cytotoxic effect of the compound **4b**was caused by the DNA damage generated by the induction of mPTP-independent apoptosis, probably by intrinsic pathway.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interests associated with the results of this study.

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REFERENCE

[1] Sherr CJ, Bartek J. Annu Rev Cancer Biol 2017;1(1):41–57.
[2] Bermedo-Carrasco S, Waldner CL. BMC Public Health2016;16:1–13.
[3] Valencia O, Lopes G, Sánchez P, Acuña L, Uribe D, González J. J Glob Oncol 2017; JGO.17.00008.
[4] DeSantis C, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, et al. CA Cancer J Clin 2014;64(4):252–71.
[5] Cragg GM, Grothaus PG, Newman DJ. Chemal Rev 2009; 109:3012–43.
[6] Dev S. Indian J Exp Biol 2010;48(3):191–8.
[7] Nirmala MJ, Samundeeswari A., Sankar PD. Res Plant Biol2011; 1(3):1–14.
[8] Mishra A, Gauri SS, Mukhopadhyay SK, Chatterjee S, Das SS, Mandal SM, et al. Peptides2014;54:148–58.
[9] Quah SY, Tan MS, Teh YH, Stanslas J. Pharmacol Ther 2016;162:35–57.
[10] Alqasoumi SI, Al-Taweel AM, Alafeefy AM, Ghorab MM, Noaman E. Eur J Med Chem2010;45(5):1849–53.
[11] Al-Said MS, Ghorab MM, Al-Qasoumi SI, El-Hossary EM, Noaman E. Eur J Med Chem., 2010, 45(7), 3011–8.
[12] Diana P, Carbone A, Barraja P, Kelter G, Fiebig H-H, Cirrincione G. Bioorg Med Chem., 2010, 18(12), 4524–9.
[13] Faidallah HM, Saqer AA, Alamry KA, Khan KA, Asiri AM. J Enzyme Inhib Med Chem 2013;1(12):12.
[14] Ghorab MM, Ragab FA, Hamed MM. Eur J Med Chem 2009;44(10):4211–7.
[15] Kovács D, Kádár Z, Mótyán G, Schneider G, Wölfling J, Zupkó I, et al. Steroids 2012;77(11):1075–85.
[16] Ratnakar Reddy K, Sambasiva Rao P, Jitender Dev G, Poornachandra Y, Ganesh Kumar C, Shanthan Rao P; et al. Bioorganic Med Chem Lett 2014;24(7):1661–3.
[17] Rudenko DA, Shavrina TV, Shurov SN, Zykova SS. Pharm Chem J 2014;108(2):100–3.
[18] Kumar KA, Jayaroopa P. Int J Pharm Chem Biol Sci. 2013;3(2):294–304.
[19] Kumbhare RM, Kosurkar UB, Janaki Ramaiah M, Dadmal TL, Pushpavalli SNCVL, Pal-Bhadra M. Bioorg Med Chem Lett 2012;22(17):5424–7.
[20] Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Mol Cancer 2015;14(1):48.
[21] Fulda S. Semin Cancer Biol 2015;31:84–8.
[22] Mosmann T. J Immunol Methods 1983;65(1–2):55–63.
[23] Noleto G.R, Petkowicz C.L.O, Merce A.L.R, Noseda M.D, Méndez-Sánchez S.C, Reicher F, Oliveira M.B.M. J Inor Biochem2009;103(5):749-757.
[24] Rodríguez Y.A, Gutiérrez M, Ramírez D,Alzate-Morales J, Bernal C.C, Güiza, FM. and Romero Bohórquez AR. ChemBiolDrug Des 2016; 88: 498-510.

[25] Bradford MM. Anal Biochem 1976;72:248–54.
[26] Appaix F, Minatchy M, Riva-Lavieille C, Olivares J, Antonsson B, Saks V a. Biochim Biophys Acta 2000;1457(3):175–81.
[27] Voss DO,. Campello AP, Bacila M. Biochem Biophys Res Commun 1961; 4 (1):48–51.
[28] Bernardi P, Vassanelli S, Veronese P, Colonna R,Szabó I,Zoratti M. J Biol Chem1992; 267 (5):2934-2939.
[29] Hou X, Luo H, Zhong H, Wu F, Zhou M, Zhang W, et al. Rapid Commun Mass Spectrom2013;27(11):1222–30.
[30] Rakesh KS, Jagadish S, Balaji KS, Zameer F, Swaroop TR, Mohan CD, et al. Inflammation2016;39(1):269–80.
[31] Palanisamy P, Jenniefer SJ, Muthiah PT, Kumaresan S. RSC Adv 2013;3(42):19300.
[32] Moger M, Pradhan A, Singh A, Govindaraju DRC, Hindupur RM, Pati HN. Med Chem Res2016;25(3):449–55.
[33] Liu J, Yu LF, Eaton JB, Caldarone B, Cavino K, Ruiz C, et al. J Med Chem 2011;54(20): 7280–8.
[34] Kouznetsov VR, Merchan Arenas D, Arvelo FS, Bello Forero J, Sojo F, Munoz A. Lett Drug Des Discov 2010;7(9):632–9.
[35] Yamasaki M, Chujo H, Koga Y, Oishi A, Rikimaru T, Shimada M, et al. Cancer Lett 2002;188(1–2):171–80.
[36] Budzisz E, Małecka M, Lorenz I, Mayer P, Kwiecien R, Paneth P, et al. Inorg Chem 2006;45(24):9688–95.
[37] Rozov FN, Grinenko TS, Levit GL, Grishakov AN, Belyavsky A V, Krasnov VP. Russ J Bioorganic Chem 2011;37(6):713–8.
[38] Jiang X, Wang X. Annu Rev Biochem 2004;73(1):87–106.
[39] Goldstein JC, Muñoz-Pinedo C, Ricci JE, Adams SR, Kelekar A, Schuler M, et al. Cell Death Differ 2005;12(5):453–62.
[40] Wang C, Youle RJ. Annu Rev Genet 2009; 43:95–118.
[41] Tait SWG, Green DR. Nat Rev Mol Cell Biol 2010;11(9):621–32.
[42] Elmore S. Toxicol Pathol 2007;35(4):495–516.
[43] Czabotar PE, Lessene G, Strasser A, Adams JM. Nat Rev Mol Cell Biol2013;15(1):49–63.
[44] Ryu S.-Y, Peixoto P.M, Teijido O, Dejean L.M, Kinnally K.W. BioFactors2010; 36 (4):255-263.
[45] Nakagawa T, Shimizu S, Watanabe T,Yamaguchi O, Otsu K, Yamagata H,Inohara H, Kubo T,Tsujimoto Y. Nature2005; 434 (7033):652-658.
[46] Sang H, Zhang L, Li J. Food Chem Toxicol 2012; 50 (7): 2417-2423.
[47] Kinnally K.W, Peixoto P.M, Ryu S.Y, Dejean L.M. Biochem Biophys Acta 2011; 1813 (4): 616-622.
[48] Bonora M, WieckowskiM.R, Chinopoulos C, Kepp O, Kroemer G,Galluzzi L,Pinton P. Oncogene 2015; 34 (12):1475-1486.
[49] Mnatasakanyan N, Beutner G,Porter G.A,Alavian K.N, Jonas E.A. J Bioenerg Biomembr 2017; 49 (1): 13-25.
[50] Mnatsakanyan N, Park H,Wu J,Miranda P,Jonas E.A. Biophysj2017; 112 (3): 440a.
[51] Nagata S. Exp Cell Res 2002;56:12–8.
[52] Zhang J, Xu M. Cell Res 2000;10:205–11.
[53] Zeng C-W, Zhang X-J, Lin K-Y, Ye H, Feng S-Y, Zhang H, et al. Mol Pharmacol 2012; 81(4):578–86.