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Dissertação

Potencial citotóxico de derivados sintéticos de quinolinas em linhagem de adenocarcinoma colorretal humano

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Potencial citotóxico de derivados sintéticos de quinolinas em linhagem de adenocarcinoma colorretal humano

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Resumo

KABKE, Augusto. **Potencial citotóxico de derivados sintéticos de quinolinas em linhagem de adenocarcinoma colorretal humano.** 2015. 47fs. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Quinolinas são compostos nitrogenados heterocíclicos compostos por um anel benzeno fusionado a pirimidina. Estão presentes em diversas substâncias naturais, principalmente alcaloides derivados de plantas e compostos sintéticos. Inicialmente utilizados com antimalárico, hoje descreve-se muitas outras atividades biológicas como antituberculose, antibacteriana, antifúngica, antioxidante, anti-inflamatória e antidepressiva. Além disso, muitos compostos que apresentam anéis de quinolina em sua estrutura possuem atividade tumoral. Esta propriedade é alvo de interesse no desenvolvimento de novos fármacos no combate ao câncer.
No presente estudo, os novos derivados de quinolinas 1-(7-cloroquinolin-4-il)-5-metil-N-fenil-1H-1,2,3-triazol-4-carboxamida (QTCA1), 1-(7-cloroquinolin-4-il)-N-(4-fluorofenil)-5-metil-1H-1,2,3-triazol-4-carboxamida (QTCA4) e 7-cloro-4-(fenilselanil) quinolina (QSPH) foram avaliados quanto ao potencial citotóxico e capacidade de induzir apoptose em linhagem celular de adenocarcinoma colorretal humano (HT-29). Os compostos foram testados sozinhos e em combinação com 5-fuorouracil (5-FU). Das substâncias testadas, a QSPH mostrou potencial citotóxico sendo capaz de inibir o crescimento celular. Combinações de derivado de quinolina QTCA4 com 5-FU aumentaram a citotoxicidade do fármaco.

Palavras-chave: câncer colorretal, HT-29, quinolina, 5-FU

Abstract

KABKE, Augusto. **Cytotoxic potential of synthetic quinolines derivatives in human colorectal cancer cell**. 2015. 47fs. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Quinolines are heterocyclic nitrogen compounds consisting of a benzene ring fused to a pyrimidine. They are present in many natural substances, especially in plants alkaloids and synthetic compounds. Initially used as antimalarial drug, today present many other biological activities: tuberculosis, antibacterial, antifungal, antioxidant, antiinflammatory and antidepressant. Furthermore, many compounds that show quinoline rings in its structure have anticancer activity. This property is interesting for the development of new drugs to combat cancer.
In this study, new derivatives of quinolines 1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazole-4-carboxamide (QTCA1), 1-(7-Chloroquinolin-4-yl)-*N*-(4-fluorophenyl)-5-methyl-1*H*-1,2,3-triazole-4-carboxamide (QTCA4) and 7-chloro-4-(phenylselanyl)quinoline (QSPH) were evaluated for cytotoxic potential and ability to induce apoptosis in human colorectal adenocarcinoma cell line (HT-29). The compounds were tested alone and in combination with 5-fluorouracil (5-FU). QSPH showed potent cytotoxic effect inhibiting cell growth. Combinations of quinoline derivative QTCA4 with 5-FU enhanced drug cytotoxicity.

Keywords: colorectal cancer, HT-29, quinoline, 5-FU.

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Lista de Abreviaturas

5-FU – 5-fluorouracil

EGFR – receptor do fator de crescimento epidérmico

VEGF – fator de crescimento endotelial

Sumário

1. INTRODUÇÃO GERAL

Câncer Colorretal

Segundo o projeto Globocan 2012 da Organização Mundial da Saúde, houve mais de 14 milhões de novos casos de câncer e 8 milhões de óbitos no mundo em 2012 (Globocan, 2012). É a segunda causa mais comum de mortes nos Estados Unidos e é esperado que ultrapasse as doenças cardiovasculares nos próximos anos como a causa mais frequente. Estima-se 1.658.370 novos casos de câncer nos Estados Unidos em 2015, com 589.430 mortes (Siegel et al., 2015). No Brasil em 2014, estima-se que houve 395.000 novos casos de câncer (INCA 2014).

O câncer colorretal foi responsável pela morte de 694 mil pessoas no mundo em 2012 (Globocan, 2012). No Brasil é o 3º tipo mais comum de câncer entre os homens e o 2º tipo entre as mulheres com mais de 32.000 novos casos em 2014 (INCA, 2014).

Atualmente, o tratamento do câncer de cólon é baseado em cirurgia e uso de quimioterapia conforme estadiamento da doença. Segundo a AJCC (American Joint Committee on Cancer), o câncer de cólon é dividido em quatro estágios conforme informações clinico-patológicas. Pacientes em estádio I e II são aqueles com doença limitada ao cólon com diferença apenas na profundidade da invasão mural. Enquanto indivíduos que apresentem metástases em linfonodos regionais pertencem ao estágio III e indivíduos com metástases em órgãos distantes pertencem ao estágio IV. Aproximadamente metade dos pacientes apresentará envolvimento linfonodal no momento da cirurgia. Além disso, 30% dos pacientes operados apresentarão metástases hepáticas não visualizadas na cirurgia e 50% dos pacientes terão recidiva hepática após a ressecção das metástases (Rossi & Rothenberger, 2006). Os indivíduos com adenocarcinoma de cólon em estágios III e IV e alguns em estágio II que apresentarem fatores de risco serão submetidos a tratamento quimioterápico (NCCN 2015).

O 5-fluorouracil (5-FU) é o principal quimioterápico utilizado no tratamento do câncer de cólon. É uma droga antimetabólito que exerce seu efeito através da inibição da enzima timidilato sintase e pela incorporação de seus metabólitos ao RNA e DNA (Longley et al., 2003). O uso de quimioterapia com base no 5-FU aumentou a

sobrevida global e sobrevida livre de doença dos pacientes com câncer colorretal ressecado em estágio III (IMPACT, 1995). Entretanto, a monoterapia com 5-FU para casos avançados tem uma taxa de resposta de apenas 15% (Johnston & Kaye, 2001). Diversas estratégias vêm sendo utilizadas para aumentar esta taxa de resposta. A associação com Leucovorin (5-formiltetrahidrofolato) é usada para aumentar as taxas intracelulares de folato reduzido necessário para a ligação dos metabólitos do 5-FU a timidilato sintase. Com isso, ocorre um incremento de 11% para 23% na taxa de resposta mas sem efeito na sobrevida global (Longley et al., 2003). A partir do uso combinado de irinotecano ou oxaliplatina com 5-FU, houve melhora nas taxas de resposta para mais de 40% em pacientes com doença metastática (Giacchetti et al., 2000; Douillard et al., 2000). O irinotecano atua na inibição da enzima topoisomerase I, enquanto a oxaliplatina é um agente de dano ao DNA (Longley et al., 2003).

Anticorpos monoclonais também podem ser utilizados em associação ao esquema de quimioterapia convencional no tratamento de pacientes com doença metastática. A adição de bevacizumabe, que bloqueia a ação do fator de crescimento endotelial - VEGF, ao esquema combinado resultou em aumento da sobrevida global, sobrevida livre de doença e taxas de resposta quando comparado ao esquema com 5-FU e irinotecano (Hurwitz et al., 2004) ou 5-FU e Leucovorin (Hurwitz et al., 2005). Em pacientes com doença metastática e com o gene *RAS* selvagem, há uma tendência ao benefício no uso de cetuximabe, anticorpo contra o receptor de fator de crescimento epidérmico – EGFR, (Van Cutsem et al., 2015) e panitumumabe, anticorpo anti-EGFR totalmente humanizado (Douillard et al., 2013).

Quinolinas: uma estratégia biotecnológica

As quinolinas (Fig.1) são compostos heterocíclicos aromáticos nitrogenado caracterizados por uma estrutura de anel duplo contendo um anel benzeno fusionado a piridina. Foram descobertas por Gerhardt em 1842 como resultado da decomposição de quinina (Manske, 1942). A estrutura heterocíclica nitrogenada das quinolinas está presente em diversos composto naturais, especialmente em alcaloides (Wilhelm et al., 2014).

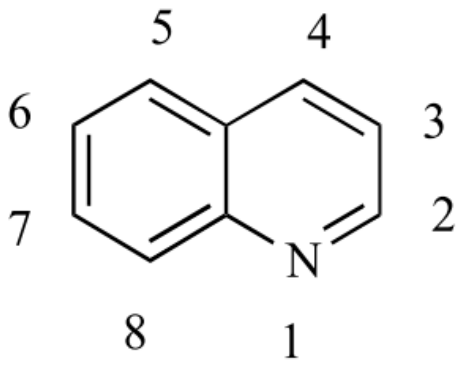

Figura 1 - Estrutura da quinolina

Devido a sua presença na composição de diversas substâncias naturais e produtos comerciais como fármacos, fragrâncias e tinturas, as quinolinas e seus derivados têm sido objeto de interesse e de estudo ao longo dos anos (Solomon & Lee, 2011; Abonia et al., 2012).

A atividade biológica das quinolinas não depende apenas de seus anéis aromáticos. A presença, a natureza e a relação espacial dos diversos substituintes periféricos confere diferentes tipos e potenciais ativos possibilitando o amplo espectro de propriedades farmacológicas destes derivados (El-Agrody et al., 2012).

Diversos estudos comprovam a variada atividade dos derivados de quinolinas. Desde os anos 40, o potencial antimalárico destes compostos é conhecido com o uso da cloroquina (Vandekerckhove & Matthias, 2015). Além disso, possuem propriedades farmacológicas que resultam em efeitos anti-inflamatórios, hipoglicemiantes, anti-hipertensivos, antiasmáticos, anti-histamínicos e antidepressivos (Wilhelm et al., 2014). Outros estudos documentam atividades antibacteriana (Ferretti et al., 2014),

antituberculose (Candéa et al., 2009) e antifúngica (Musiol et al., 2006). Entretanto, o maior interesse atual é no desenvolvimento de moléculas para o combate ao câncer. As quinolinas são a base para compostos que apresentam atividade antitumoral *in vitro* comprovada por vários estudos (Sun et al., 2013; Tang et al., 2013).

Diversos mecanismos de ação antitumoral foram descritos para as quinolinas e seus análogos: inibição de tirosina quinases c-Met (Tang et al., 2014; Zhou et al., 2014), proteossoma, polimerização de tubulina e reparo de DNA (Solomon & Lee 2011). Além destes, outro mecanismo de funcionamento é a inibição da enzima topoisomerase I pelo alcaloide natural Camptotecin e seu análogo sintético Topotecano (Arafa et al., 2013).

Alguns estudos relatam que derivados de quinolinas foram capazes de reverter resistência a múltiplas drogas quimioterápicas mediada pela expressão aumentada de glicoproteína P. Esta proteína atua como uma bomba de efluxo diminuindo a concentração intracelular dos fármacos (Ganguly et al., 2011; Arafa et al., 2013; Karthikeyan et al., 2015).

Assim, esta dissertação apresenta os resultados de estudos com derivados de quinolinas em linhagem celular de adenocarcinoma colorretal na forma de dois artigos científicos.

2. HIPÓTESE E OBJETIVOS

2.1. Hipótese

Derivados sintéticos de quinolinas têm ação antitumoral em linhagem celular de adenocarcinoma colorretal humano.

2.2. Objetivo Geral

Identificar novos fármacos derivados de quinolinas com potencial antitumoral, avaliando os mecanismos de ação envolvidos e a toxicidade celular destes compostos.

2.3. Objetivos Específicos

- Avaliar o potencial antiproliferativo de derivados de quinolinas 1-(7-cloroquinolin-4-il)-5-metil-N-fenil-1H-1,2,3-triazol-4-carboxamida (QTCA1), 1-(7-cloroquinolin-4-il)-N-(4-fluorofenil)-5-metil-1H-1,2,3-triazol-4-carboxamida (QTCA4) e 7-cloro-4-(fenilselanil) quinolina (QSPH) em linhagem celular de adenocarcinoma colorretal humano (HT-29);
- Verificar as alterações morfológicas em células HT-29 frente aos derivados de quinolinas;
- Avaliar o grau de apoptose das células tumorais frente aos derivados de quinolinas;
- Avaliar a expressão de genes envolvidos no processo de apoptose;

3. ARTIGO 1

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para Prelimminary Communication

A Quinoline Derivative 1-(7-Chloroquinolin-4-yl)-*N*-(4-fluorophenyl)-5-methyl-1*H*-1,2,3-triazole-4-carboxamide increases 5-Fluorouracil cytotoxicity in human colorrectal cancer cells

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Abstract

Quinoline is widely used in chemistry as scaffold to biologically active compounds. The *in vitro* antiproliferative activity of 1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazole-4-carboxamide (QTCA1) and C (QTCA4) was tested against human colorectal cancer cell line HT-29. We also assess a possible interaction between these compounds and 5-fluorouracil. QTCA4 enhanced the cytotoxic effect of 5-FU but the apoptotic pathway involved in the cellular death remains unclear.

Keywords: colorectal cancer; quinoline; 5-fluorouracil; HT-29

Introduction

It is estimated that cancer is the second leading cause of death in United States and is expected to exceed cardiovascular disorders as leading cause of death in the next years [1].

Colorectal cancer was responsible for almost 700.000 deaths worldwide in 2012 [2]. For 2015, there is an estimative of more than 130.000 new cases in the United States with 49.700 deaths related to colorectal cancer [1].

Although the combination of 5-fluorouracil (5-FU) with irinotecan and oxaliplatin has improved the response and survival rates of patients with advanced colorectal cancer [3][4], only 30-50% of metastatic patients will have a satisfactory outcome. One cause to treatment failure is drug resistance [5]. This fact made the discovery of new chemotherapeutic agents capable of overcome drug resistance without toxicity a great challenge to medicinal chemistry.

Used since 1940 as antimalarial drug [6], quinolines structure are present in many natural products, mostly in many different plants [7]. They have several biological and pharmacological proprieties described such as: antitubercular [8], antibacterial[9], antioxidant [10][9], antidepressant, anti-hypertensive, anti-inflammatory, hypoglycemic [11] and antifungal [12]. One of the most studied pharmacological proprieties of quinoline derivatives is anticancer activity with several studies proving the effectiveness of compound with quinoline ring against cancer cells [13][14][15].

The present study will access the cytotoxicity and involved pathways of compounds combining quinoline rings and 1,2,3-triazole derivative. 1,2,3-triazole has already shown their antitumoral effects. Some 1,2,3-triazole based compounds are under clinical trial for cancer therapy [16].

Results and Discussion

Chemistry

The synthesis of quinolines derivatives 1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazole-4-carboxamide (QTCA1) and 1-(7-Chloroquinolin-4-yl)-*N*-(4-fluorophenyl)-5-methyl-1*H*-1,2,3-triazole-4-carboxamide (QTCA4) was described in previously study (Fig.1) [11].

Cytoxicity and Biological Activity

The *in vitro* cytotoxicity of QTCA1, QTCA4 and commercial quinoline was assessed against human colorectal cancer cell line HT-29 by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide] assay. The compounds were tested alone and combined with 5 μM of 5-fluorouracil (5-FU) as described previously [17]. Cell incubation with QTCA1 and QTCA4 showed slightly none inhibition rate at 24, 48 and 72 hours of treatment (Fig. 2).

However, when combined with 5 μM of 5-FU, QTCA4 apparently enhanced the cytotoxic effect of 5-FU at 48 and 72 hours. The inhibition rate of 5-FU alone was close to 20% in our study and the combination of 5-FU with 20 μM of QTCA4 showed inhibition rate of 45% (Fig. 3). The combination of 5-FU with commercial quinoline showed no statistical difference when compared to isolated treatments. Since QTCA1 did not present cytotoxicity, we decided not to test it in combination with 5-FU or use it in other assays.

In order to assess the cell viability, we used LIVE/DEAD (Invitrogen Life Technology) with DAPI (Invitrogen Life Technology) counterstaining. The assay was performed after 48 hours of HT-29 incubation with tested compounds and associations.

Cells treated with 5 μM of 5-FU alone showed almost 40% of apoptotic cells. After 48 hours of treatment with 10 μM and 20 μM of QTCA4 and commercial quinoline, the number of live and apoptotic cells was similar to control. When combined with 5-FU, QTCA4 and commercial quinoline promoted a decrease in live cell percentage compared to control (FIG. 4).

We also assess HT-29 gene expression profile for apoptotic pathway (Bax, Bcl-2, Caspase 3, 8 and 9). Cells were again exposed to 5 μM of 5-FU, 20 μM of QTCA4, 20 μM of commercial quinoline and this concentrations combined to 5-FU. They were incubated for 48 hours. Bax, bcl-2, Caspase 3 and Caspase 9 had their expression decrease compared to negative control ($P<0.05$). No difference between 5-FU, quinolines and combined treatments was found in this study (Fig. 5).

Cells treated with 5-FU and quinoline derivatives combined with 5-FU showed an increase Bax/Bcl-2 ratio compared to control.

Conclusion

In the present study, we assess antitumoral effect of synthetic quinoline derivatives. QTCA-4 was able to improve the cytotoxic effect of an already established drug in treatment of human colorectal cancer (5-FU). Using low concentrations of both compound combined, the growth inhibition of HT-29 was closely to 50%. However, we are not able to determine the pathway involved in cellular death. More assays will be done to elucidate those pathways and compounds action.

We believe that these quinoline derivatives have a great potential as a template for new anticancer drugs.

Experimental Protocols

Cellular Culture

The human colon cancer cell lines HT-29 were purchased from Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). This tumor cell line was cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum - FBS (Gibco, USA) and maintained at 37°C, 95%

humidified air and 5% CO_2 (Force® Heal, China) until they reached sub-confluence stage (~90%). The experiments were performed with cells in the logarithmic phase of growth and all experiments were run in triplicate.

Cellular proliferation assay

The cytotoxicity was determined by MTT assay. The cell suspension was prepared at a density of 5 x 10^4 per well and distributed into a 96 well-plate. After plating, cells were incubated at 37°C, 95% humidified air in 5% CO_2 for 24 h. Following this, cells were treated with different concentrations of compound QTCA1, QTCA4 and commercial quinoline (5, 10, 20, 40 and 80 µM) and 5-fluorouracil (2.5, 5, 10, 20 and 40 µM). Plates were incubated for 24 h, 48 h and 72 h. After incubation periods, experimental materials were removed and subsequently 180 µL of medium and 20 µL MTT (5 mg MTT/mL solution) was added to each well. The plates were incubated for an additional 3 h at 37°C. Differences in total cellular metabolism were detected at a wavelength of 492 nm using a microplater reader. The inhibition (%) of cell proliferation was determined as follows: inhibitory growth = $(1-Abs_{492treated}$ cells/$Abs_{492control}$ cells) × 100%. The same assay using combinations of 5 µM 5-fluorouracil and quinoline derivatives was performed.

Live/Dead assay and DAPI counterstaning

The LIVE/DEAD cell viability assay® (Invitrogen™, Carlsbad, CA, USA) was performed in accordance with manufacturer's instructions. Live cells were able to take up calcein and could be analyzed by green fluorescent light emission (488 nm). Ethidium bromide homodimer diffuses through the permeable membrane of dead cell and binds into their DNA. Red fluorescent signal (546 nm) detected dead. The LIVE/DEAD assay was analyzed with a fluorescence microscope Olympus IX71 (Olympus Optical Co.) by multicolor imaging. The fluorescence emissions were photographed using a digital camera (Nikon, Tokyo, Japan) attached to a fluorescence microscope (DP 12; BX 51; Olympus, Tokyo, Japan). Cell^F software (Cell-F, New York, USA) analyzed the recorded images. The data were expressed as mean ± SEM of 2 observations in different fields with 300 cells in each field. After 20 minutes of

incubation, DAPI (4',6-diamidino-2-phenylindole) was added to cells and photographed according to manufacturer's protocol without fixing cells.

Quantitative Real-Time PCR

Cells were added in a 6-well plate at a density of 1 x 10^6 /mL per well and grown at 37°C, 95% humidified air in 5% CO_2 for 24 h. The cells were treated with 1 mL of QTCA4 and commercial quinoline at concentrations of 20 µM and in combination with 5-fluorouracil (5 µM). The total RNA extracted and cDNA was synthesized as other studies [18]. The RNA samples were isolated using TRIzol® Reagent (Invitrogen™, Carlsbad, USA) and treated with DNA-free® kit (Ambion™, USA) in accordance with manufacture's protocol. cDNA synthesis was performed with 1 µg of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) following manufacture´s protocol. Real-time PCR reactions were performed on a Stratagene® Mx3005P™ Real-Time PCR System (Agilent Technologies, USA) using SYBR® Green PCR Master Mix (Applied Biosystems™, UK) and primers described in table.

Data Analysis

Data sets were analyzed using one-way or two-way ANOVA followed by a Tukey test for multiple comparisons. Significance was considered at $P<0.05$ in all analyses. Data were expressed as mean ± SEM.

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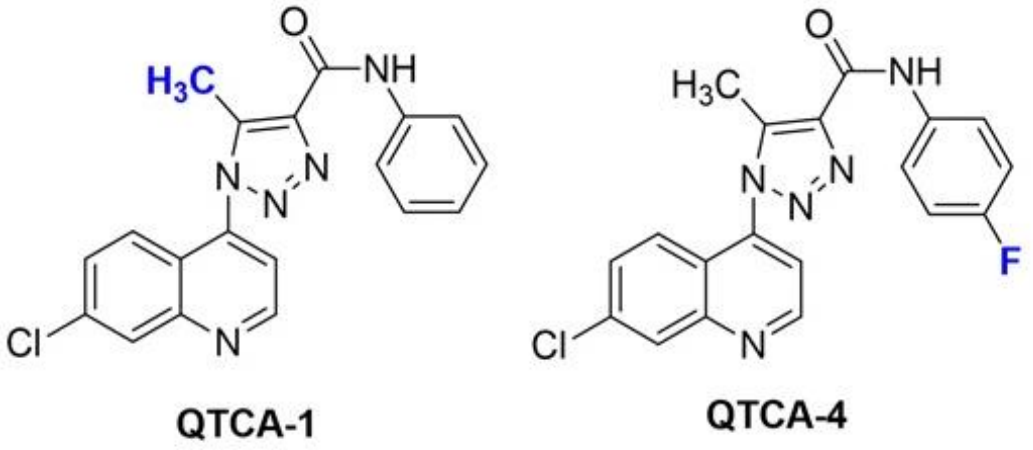

Figure 1 - QTCA1 (1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazole-4-carboxamide) and QTCA4 (1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazole-4-carboxamide) structures.

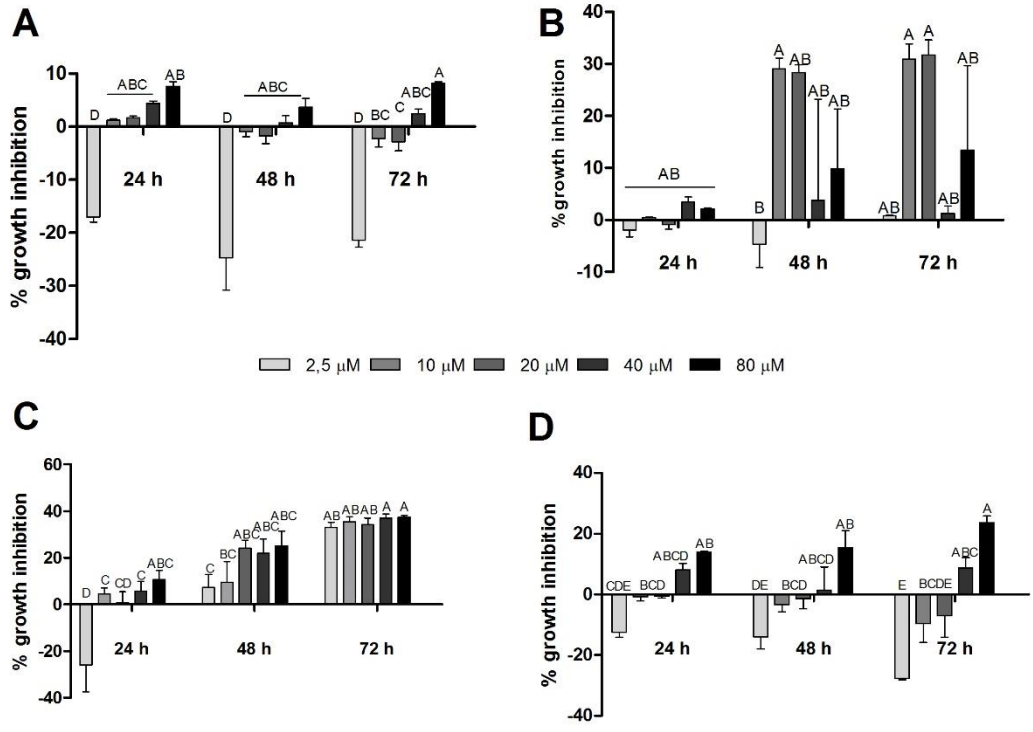

Figure 2 - Cytotoxic effect of quinoline derivative in colorectal cancer cell line HT-29. Cells treated with QTCA1 (A), commercial quinoline (B), 5-FU (C) and QTCA4 (D). Different letter above each column represents statistical difference.

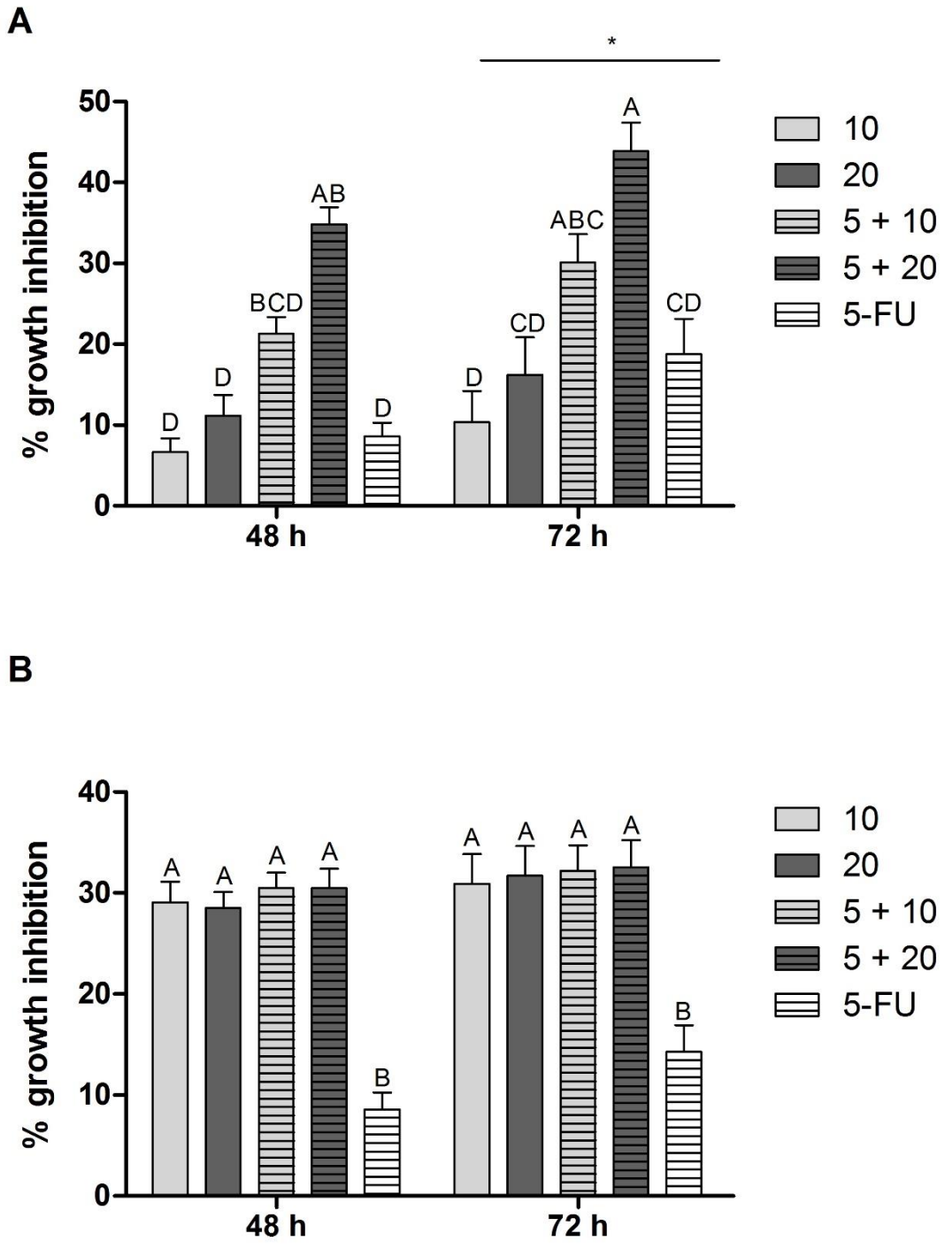

Figure 3 – Cytotoxic effect of combinations of 5-FU with QTCA4 (A) and commercial quinoline in colorectal cancer cell line HT-29 (B). Different letters above columns indicate statistical significant difference.

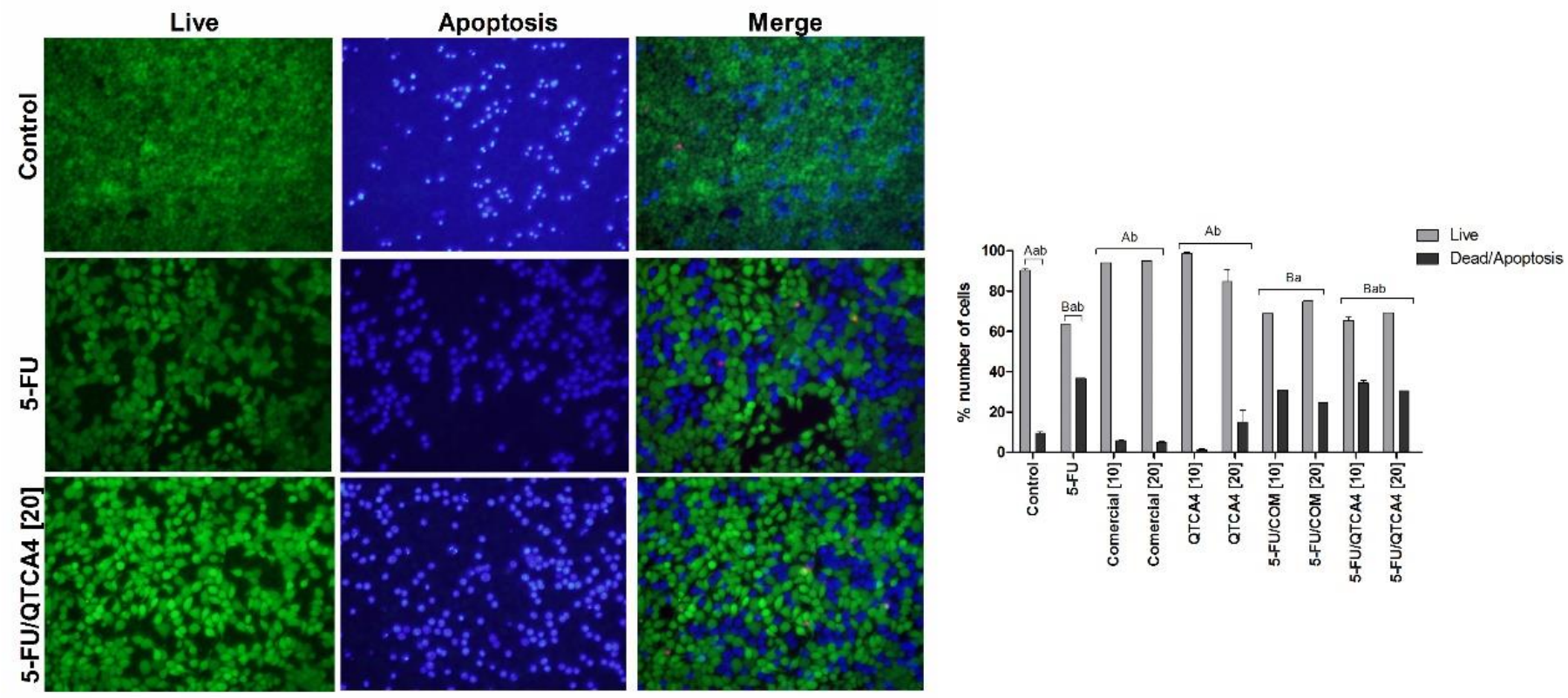

Figure 4 – Colorectal cancer cells were treated with 5-FU, QTCA4 and 5-FU combined to QTCA4. Quantitative analysis of apoptotic cells was estimated by LIVE/DEAD assay with DAPI counterstaining.

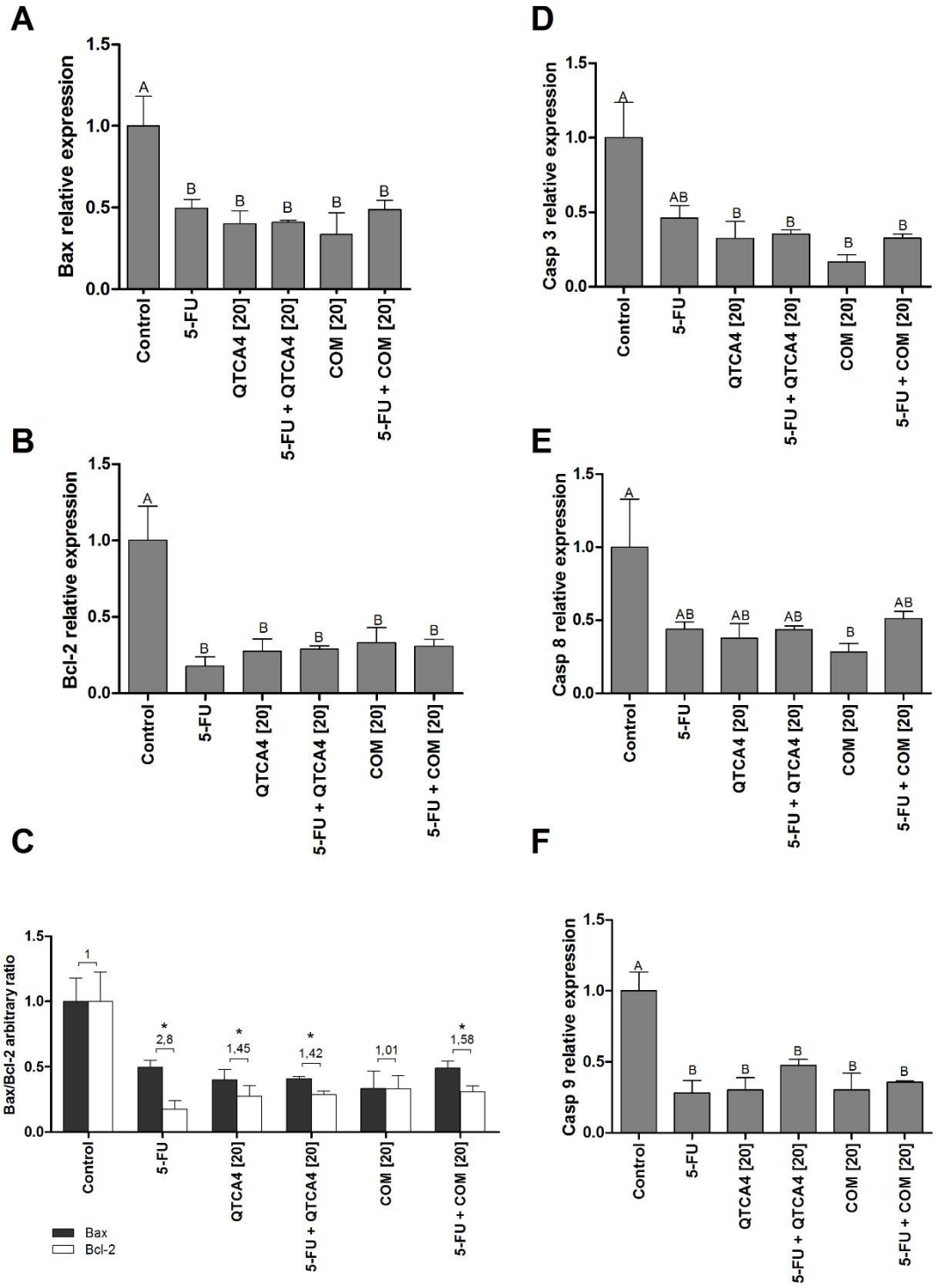

Figure 5 – HT-29 gene expression profile after 48h of incubation with 5-FU, QTCA4, commercial quinoline and combinations, RNA was extracted and cDNA synthesized. Real Time PCR was performed to analyze Bax (A), Bcl-2 (B), Bax/Bcl-2 ratio (C), Caspase 3 (D), Caspase 8 (E) and Caspase 9 (F) expression.

Table 1. Primers used in the study

Primers	**Sequence 5'→ 3'**
Bcl-2 For	GTGTGGAGAGCGTCAACC
Bcl-2 Rev	CTTCAGAGACAGCCAGGAG
Bax For	ATGCGTCCACCAAGAAGC
Bax Rev	ACGGCGGCAATCATCCTC
Caspase-3 For	CAGTGGAGGCCGACTTCTTG
Caspase-3 Rev	TGGCACAAAGCGACTGGAT
Caspase-8 For	GGATGGCCACTGTGAATAACTG
Caspase-8 Rev	TCGAGGACATCGCTCTCTCA
Caspase-9 For	CCAGAGATTCGCAAACCAGAGG
Caspase-9 Rev	GAGCACCGACATCACCAAATCC
GAPDH For	GGATTTGGTCGTATTGGG
GAPDH Rev	TCGCTCCTGGAAGATGG

4. ARTIGO 2

Este manuscrito foi formatado conforme as normas do periódico

European Journal of Medicinal Chemistry

para Prelimminary Communication

Antitumoral effect of 7-chloro-4-(phenylselanyl)quinoline in human colorectal cancer cell line

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Abstract

Anticancer proprieties of quinoline derivatives are extensively decribed. As well as the biological activities of diphenylselenide are known. In this study, we assessed the *in vitro* antitumoral behavior of 7-chloro-4-(phenylselanyl) quinoline (QSPH) in human colorectal cancer cell line HT-29 and a possible interaction with 5-fluorouracil. QSPH alone was able to inhibit cellular growth by 65% and enhanced the *in vitro* cytotoxicity of 5-FU. Since the compound has strong anticancer proprieties more studies are need to determine the precise dead pathway involved in this phenomenon.

Keywords: colorectal cancer; quinoline; HT-29; 5-fluorouracil; selenium

Introduction

According to Globocan project from World Health Organization, there was 14 million new cases of cancer in 2012 and 8 million deaths [1]. In United States of America, it is the second leading cause of death [2]. Colorectal cancer was responsible for 694.000 deaths worldwide in 2012 [1].

At least, 50% of patients submitted to surgery for colon cancer have lymphatic spread and need adjuvant chemotherapy [3]. Despite combined treatment using 5-fluororacil and oxaliplatin or irinotecan had significantly improved the response rate and survival of metastatic colorretal cancer patients [4,5], new strategies and drugs are required. Only 30% to 50% of metastatic patients undergoing in chemotherapy with this combinations will have a satisfactory response. The treatment fails were probably due to drug resistance [6]. The development of new chemotherapeutic agents that overcomes resistance without excessive toxicity is a challenge to medicinal chemistry [7].

Quinoline ring system is a versatile scaffold to biologically active compounds. It is present in many natural products and their derivatives have many varieties of proven pharmacological proprieties: anti-inflammatory, anti-asthma, hypoglycemic, anti-hypertensive, antidepressants [8], antibacterial [9], antitubercular [9], antifungal [10] and antimalarial [11]. However, the most interesting feature in these compounds is their anticancer activity and several studies had showed this propriety [7,12,13].

The antioxidant [14], neuroprotective, antinociceptive [15] and antidepressant [16] proprieties of diphenyldiselenide had been described. Diphenyldiselenide has also shown anticancer activity in human neuroblastoma cells [17], however it was not cytotoxic when tested in human colorectal cancer cell line HT-29 [18].

In the present study, we investigate the cytotoxic propriety of a derivative 7-chloro-4-(phenylselanyl) quinoline in human colorectal cancer cell line and attempt to study the underlying cell death mechanisms.

Results and Discussion

Synthesis of Quinoline Derivative

The synthesis of 7-chloro-4-(phenylselanyl) quinoline (QSPH) (Fig. 1) was made by the reaction of 4,7-dichloroquinoline with diphenyl diselenide as described in a previously study [19].

Biological Activity

We used MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide] assay to determine *in vitro* cytotoxicity of QSPH in HT-29 cells . This quinoline derivative was tested alone and in combination with 5-FU. Different concentrations of QSPH were combined to 5 µM of 5-FU as used previously [20,21]. QSPH showed a time related cytotoxicity. The maximum growth inhibition was 6.81% and 56.72% in 24 hours and 72 hours respectively with a concentration of 40 µM. Furthermore, the inhibition rate was dose-dependent after 72 hours of incubation with 20 µM, 40 µM and 80 µM showing 49.51%, 56.72% and 65.52% of inhibition rate respectively (Fig. 2).

A combined treatment of QSPH and 5 µM of 5-FU, exhibited a decrease growth inhibition rate when compared to QSPH alone. However, the association was able to increase 5-FU cytotoxicity. Treatment with 5 µM of 5-FU alone showed less than 20% of growth inhibition while combined with 10 µM and 20 µM of QSPH was able to inhibit closely to 40% of cellular growth (Fig. 3).

The cellular viability was determined by using LIVE/DEAD with DAPI counterstaining. The test was performed and photographed after 48 hours of incubation with QSPH alone and combined to 5-FU. No difference was observed in percentage of apoptotic cells after exposure after exposure to 5 µM of 5-FU when compared to control. On the other hand, treatment with 10 µM and 20 µM of QSPH alone resulted in 30% and 60% of apoptotic cells respectively compared to control. Combinations of QSPH and 5-FU showed significantly enhanced the percentage of apoptosis showing apoptotic rates up to 45% (Fig. 4).

In order to determine what apoptotic pathways were involved in cellular death, we assess the HT-29 gene expression profile. Despite QSPH have shown considerable cytotoxicity, there was no increment in HT-29 expression of proapoptotic pathways investigated in this study. Cells exposed to QSPH alone and combined to 5-FU showed a decreased in expression of all apoptotic pathways compared to negative control. The exception was Bcl-2 expression in cells treated with 20 µM of QSPH exhibited an increase 2-fold.

Nevertheless the apoptotic gene expression pattern in cells exposed to QSPH was similar to those treated with 5-FU. Exception is made to Bax/Bcl-2 ratio where cells treated with 5 µM of 5-FU had an increase in this ratio compared to control (Fig. 5). We believe that cellular death occurs through another apoptotic pathway different from caspase.

Conclusion

Despite diphenylselenide had not showed cytotoxicity against HT-29, in the present study when linked to quinoline rings appeared to be a potential cytotoxic agent. Besides, it was able to significantly improve in *in vitro* cytotoxic effect of a clinical consecrated drug, 5-fluorouracil.

More studies are needed and will be done to determine the pathways and mechanisms involved in the cytotoxic effect of this compound and its real interaction with 5-FU.

Experimental Protocols

Cellular culture

The human colon cancer cell lines HT-29 were purchased from Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). This tumor cell line was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum - FBS (Gibco, USA) and maintained at 37°C, 95% humidified air and 5% CO_2 (Force® Heal, China) until they reached sub-confluence stage (~90%). The experiments were performed with cells in the logarithmic phase of growth and all experiments were run in triplicate.

Cellular proliferation assay

Cell cytotoxicity after QSPH treatment was determined by MTT assay. The cell suspension was prepared at a density of 5 x 10^4 per well and distributed into a 96 well-plate. After plating, cells were incubated at 37°C, 95% humidified air in 5% CO_2 for 24 h. Following this, cells were treated with different concentrations of QSPH compound (5, 10, 20, 40 and 80 µM), 5-fluorouracil (2.5, 5, 10, 20 and 40 µM) and combinations of 5-fluorouracil and QSPH (5 µM of 5-FU combined with 10 or 20 µM of QSPH). Plates were then incubated for 24 h, 48 h and 72 h. After incubation periods, experimental materials were removed and subsequently 180 µL of medium and 20 µL MTT (5 mg MTT/mL solution) was added to each well. The plates were incubated for an additional 3 h at 37°C. Differences in total cellular metabolism were detected at a wavelength of 492 nm using a microplater reader. The inhibition (%) of cell proliferation was determined as follows: inhibitory growth = $(1-Abs_{492treated}$ cells/$Abs_{492control}$ cells) × 100%.

Live/Dead assay and DAPI counterstaning

The LIVE/DEAD cell viability assay® (Invitrogen™, Carlsbad, CA, USA) was performed in accordance with manufacturer's instructions. Live cells were able to take up calcein and could be analyzed by green fluorescent light emission (488 nm). Ethidium bromide homodimer diffuses through the permeable membrane of dead cell

and binds into their DNA. Red fluorescent signal (546 nm) detected dead. The LIVE/DEAD assay was analyzed with a fluorescence microscope Olympus IX71 (Olympus Optical Co.) by multicolor imaging. The fluorescence emissions were photographed using a digital camera (Nikon, Tokyo, Japan) attached to a fluorescence microscope (DP 12; BX 51; Olympus, Tokyo, Japan. Cell^F software (Cell-F, New York, USA) analyzed the recorded images. The data were expressed as mean ± SEM of 2 observations in different fields with 300 cells in each field. After 20 minutes of incubation, DAPI (4',6-diamidino-2-phenylindole) was added to cells and photographed according to manufacturer's protocol without fixing cells.

Quantitative Real-Time PCR

Cells were added in a 6-well plate at a density of 1 x 10^6 per well and grown at 37°C, 95% humidified air in 5% CO_2 for 24 h. The cells were treated with 1 mL of QSHP at concentrations of 10 and 20 µM and in combination with 5-fluorouracil (5 µM). The total RNA were isolated using TRIzol® Reagent (Invitrogen™, Carlsbad, USA) in accordance with manufacture's protocol. cDNA synthesis was performed with 1 µg of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) following manufacture's protocol. Real-time PCR reactions were performed on a Stratagene® Mx3005P™ Real-Time PCR System (Agilent Technologies, USA) using SYBR® Green PCR Master Mix (Applied Biosystems™, UK) and primers described in table.

Data Analysis

Data sets were analyzed using one-way or two-way ANOVA followed by a Tukey test for multiple comparisons. Significance was considered at $P<0.05$ in all analyses. Data were expressed as mean ± SEM.

Acknowledgments

This work was supported by the Brazilian funding agencies CAPES and CNPq.

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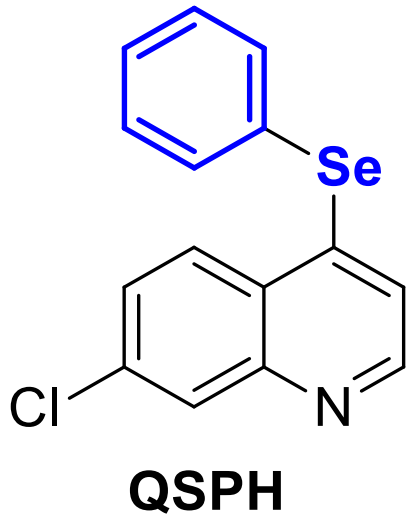

Figure 3 – QSPH (7-chloro-4-(phenylselanyl) quinolone) structure.

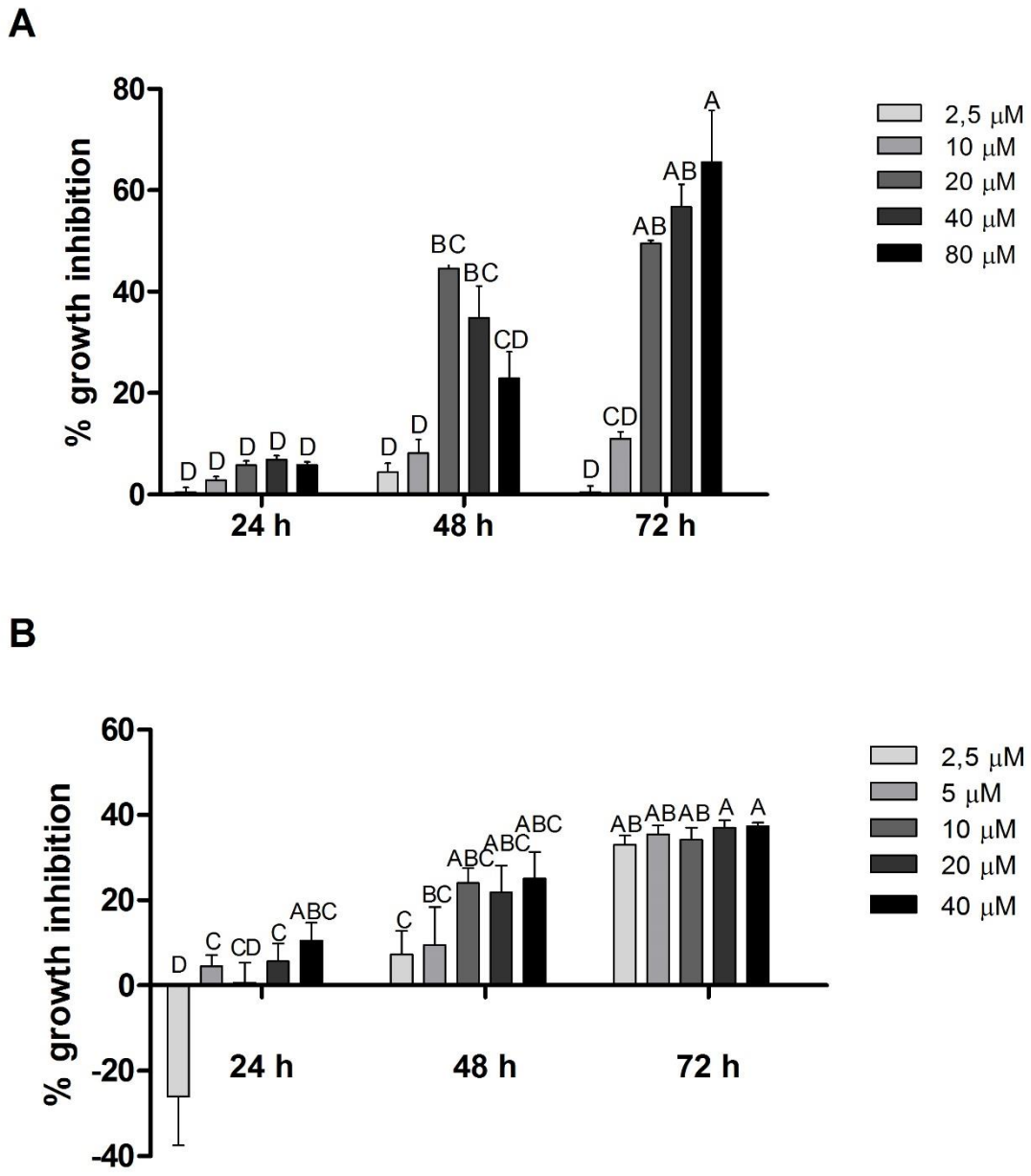

Figure 2 - Cytotoxic effect of quinoline derivative in coloretal cancer cell line HT-29. (A) Cells treated with QSPH, (B) Cells treated with 5-FU. Different letters above each column represent statistical significance.

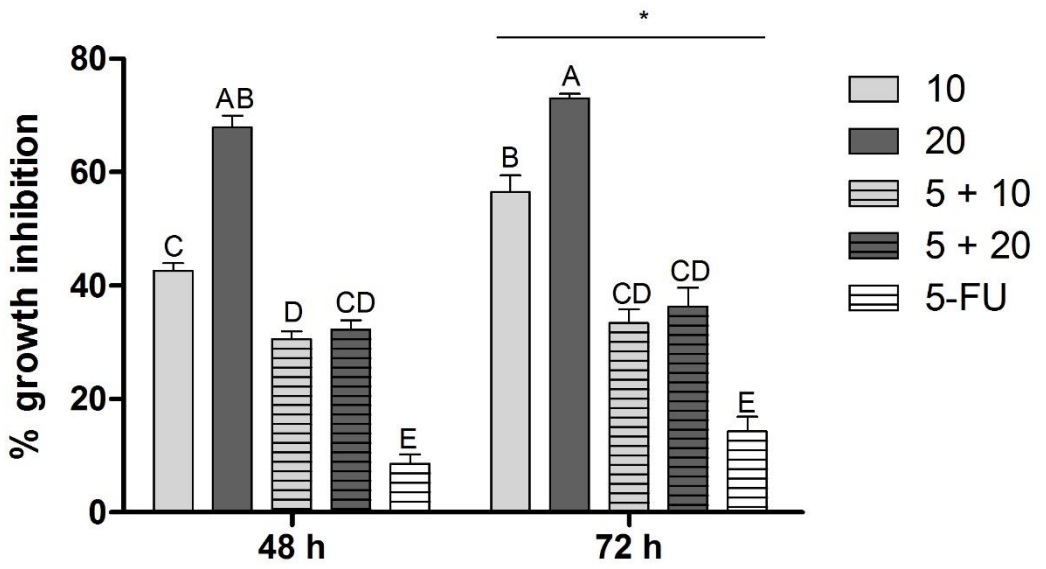

Figure 3 - Cytotoxic effect of QSPH combined with 5-FU at 48 h and 72 h in colorectal cancer cell line HT-29. Different letters above columns indicate statistical significant difference.

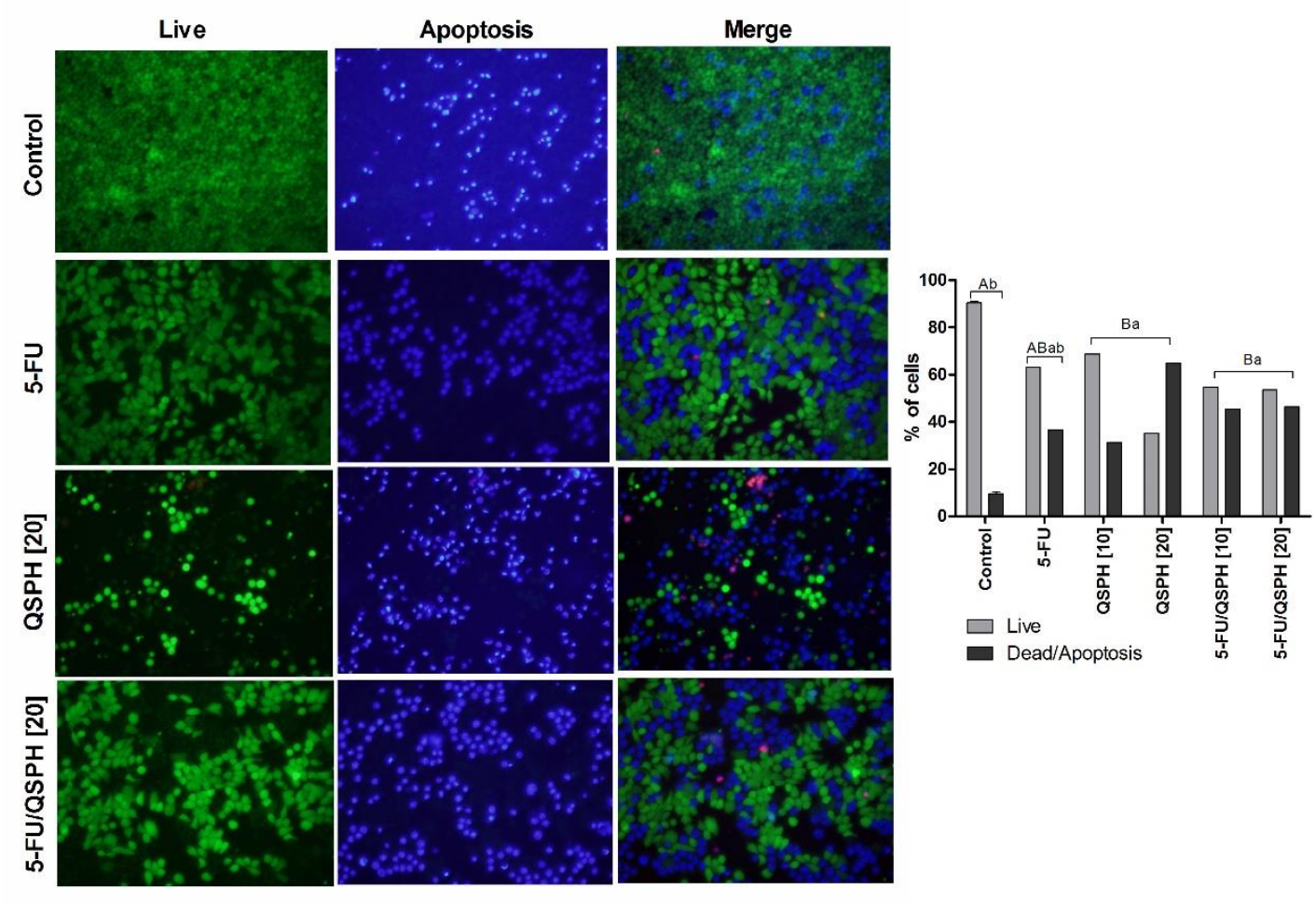

Figure 4 - Colorectal cancer cells were treated with 5-FU, QSPH and combination of 5-FU and QSPH for 48h. Quantitative analysis of apoptotic cells was estimated by LIVE/DEAD assay with DAPI counterstaining.

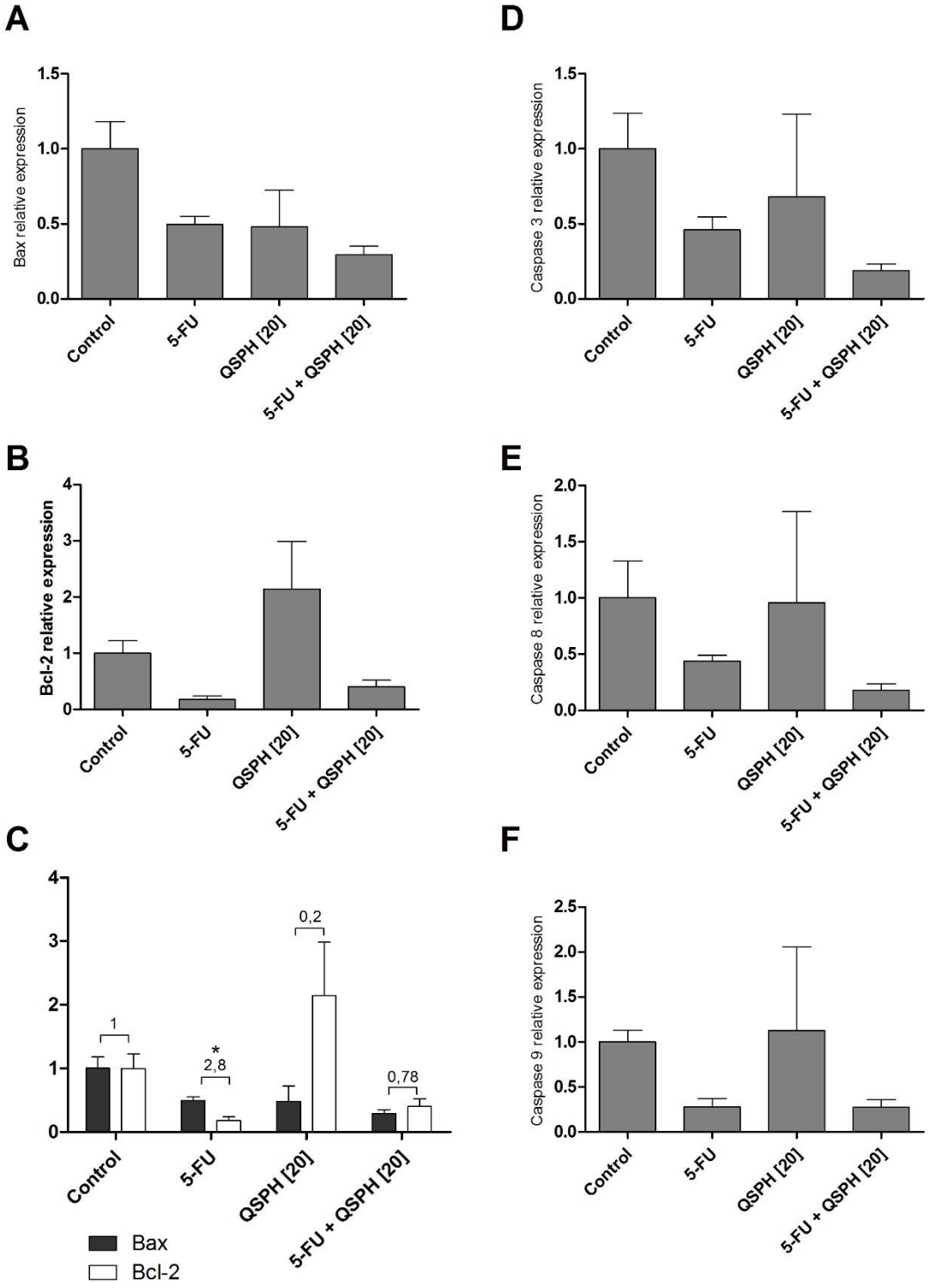

Figure 5 – HT-29 gene expression profile. After 48 h of incubation with 5-FU, QSPH and 5-FU combined with QSPH, RNA was extracted and cDNA was synthesized. Real Time PCR was performed to analyze Bax (A), Bcl-2 (B), Bax/Bcl-2 ratio (C), Caspase 3 (D), Caspase 8 (E) and Caspase 9 (F) expression. Only Bax/Bcl-2 ratio in treatment with 5-FU showed significant difference compared to control.

Table 1. Primers used in the study

Primers	**Sequence 5'→ 3'**
Bcl-2 For	GTGTGGAGAGCGTCAACC
Bcl-2 Rev	CTTCAGAGACAGCCAGGAG
Bax For	ATGCGTCCACCAAGAAGC
Bax Rev	ACGGCGGCAATCATCCTC
Caspase-3 For	CAGTGGAGGCCGACTTCTTG
Caspase-3 Rev	TGGCACAAAGCGACTGGAT
Caspase-8 For	GGATGGCCACTGTGAATAACTG
Caspase-8 Ver	TCGAGGACATCGCTCTCTCA
Caspase-9 For	CCAGAGATTCGCAAACCAGAGG
Caspase-9 Rev	GAGCACCGACATCACCAAATCC
GAPDH For	GGATTTGGTCGTATTGGG
GAPDH Rev	TCGCTCCTGGAAGATGG

5. CONCLUSÃO GERAL

No presente estudo foi testado a citotoxicidade de derivados sintéticos de quinolinas em linhagem celular HT-29 de adenocarcinoma colorretal humano. O composto QSPH mostrou potencial citotóxico *in vitro* em HT-29. Além disso, o derivado de quinolina QTCA4 quando combinado a 5-FU resultou em aumento da citotoxicidade *in vitro*. Mais estudos são necessários para elucidar possíveis rotas de apoptose e morte celular assim como os mecanismos envolvidos nesta interação entre as substâncias testadas.

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