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***Corresponding author:**
José L. Vique-Sánchez
email: jvique@uabc.edu.mx

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Potential PIK3CA Inhibitor, Advances for a Treatment against Prostate Cancer Depending on PI3K Pathway

Victor García-González, Ana Brenda Ortega Morales, Anna C. Navarro Padrón, Octavio Galindo-Hernández, José L. Vique-Sánchez*

School of Medicine Campus Mexicali, Autonomous University of Baja California, Mexicali, 21000, BC, México.

Abstract. Advances to develop new anticancer drug continue, and one of the new therapeutic targets to reach is the PI3K, due to its pathway's functions which are related to prognosis in a variety of tumors and this protein is strongly related to tumor proliferation, migration, and apoptosis by their functions on PI3K pathway. This study evaluated a potential PI3K ligand, the C5 compound, that previously our research group proposed as a compound directed to interact with the PIK3CA´s activation region to develop a drug to regulate the PI3K´s functions. The aim of this research is to evaluate the effects of the C5 compound on PI3K´s functions related to a kind of prostate cancer without androgen receptor (PC3 cell), by *in vitro* cultures.
We determined a regulation on the PI3K pathway in PC3 cell, due to a regulation in the expression of p-FAK, p-P70S6K and Caspase-3, these proteins were correlated to decrease the viability of the cultures to regulate the apoptosis, and this determined by western blot analysis. Then, the C5 compound was analyzed by molecular dynamics to describe the interactions of C5 compound with the PIK3CA´s activation region.
We propose that the C5 compound has a high probability to be selective against PI3K, with an apoptosis (regulating Caspase-3) and cytotoxic effect on prostate cancer lines (in vitro). We also describe the possible specific interactions in

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the PI3K´s activation region, related to decreasing the signaling of the PI3K pathway, and probably safe for humans. These results show the high potential of this compound as a new drug against this kind of prostate cancer.

Resumen. Continúan los avances en el desarrollo de nuevos fármacos anticancerígenos, y una de las nuevas dianas terapéuticas a alcanzar es la PI3K, debido a las funciones de su vía de señalización, que están relacionadas con el pronóstico en diversos tumores. Esta proteína está fuertemente vinculada a la proliferación, migración y apoptosis tumoral a través de sus funciones en la vía de la PI3K. Este estudio evaluó un ligando potencial de la PI3K, el compuesto C5, que nuestro grupo de investigación propuso previamente como un compuesto dirigido a interactuar con la región de activación de PIK3CA para desarrollar un fármaco que regule las funciones de la PI3K. El objetivo de esta investigación es evaluar los efectos del compuesto C5 sobre las funciones de la PI3K relacionadas con un tipo de cáncer de próstata sin receptor de andrógenos (célula PC3), mediante cultivos in vitro. Determinamos una regulación de la vía PI3K en células PC3, debido a la regulación de la expresión de p-FAK, p-P70S6K y Caspasa-3. Estas proteínas se correlacionaron con la disminución de la viabilidad de los cultivos para regular la apoptosis, lo cual se determinó mediante análisis de Western blot. Posteriormente, se analizó el compuesto C5 mediante dinámica molecular para describir sus interacciones con la región de activación de PI3K.
Proponemos que el compuesto C5 tiene una alta probabilidad de ser selectivo contra PI3K, con un efecto apoptótico (regulando la Caspasa-3) y citotóxico en líneas celulares de cáncer de próstata (in vitro). También describimos las posibles interacciones específicas en la región de activación de PI3K, relacionadas con la disminución de la señalización de la vía PI3K, y probablemente seguras para humanos. Estos resultados demuestran el alto potencial de este compuesto como un nuevo fármaco contra este tipo de cáncer de próstata.

Introduction

Prostate cancer (PCa) is the most common cancer among men, with around 1.4 million new cases diagnosed annually worldwide (Globocan 2022, WHO). The main treatments for treating PCa are about androgen deprivation therapy (ADT) and androgen receptor (AR) [1]. Some kinds of PCa become more aggressive or resistant against treatment through adaptive responses, through the AR pathway [2]. On the other hand, there are studies that showed the effects of hyperactivation of PI3K/AKT pathway in PCa, which it is important to regulate cell survival/apoptotic pathways in some kinds of cancer [3-6].

The develop of new anticancer drugs against new therapeutic targets is increasing worldwide, and we research group reported a compound against the Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit Alpha (PIK3CA) [7,8], which is a key protein in the PI3K pathway related to cell proliferation and migration, due to PI3K/AKT signaling promotes tumor cell survival, proliferation, growth, inhibition of apoptosis, and metabolism by activating its downstream effectors [3-5] and that alterations in this pathway contribute to a lack of therapeutic effects of anticancer drugs [9,10]. Particularly in prostate cancer, PIK3CA promotes invasion and tumorigenesis by several mechanisms. It promotes cell cycle progression, inhibition of apoptosis is also promoted by AKT activation of suppression of proapoptotic factors, such as BAD or BAX, enhancing anti apoptotic factors, thus promoting cell survival. When it comes to tumorigenesis, PIK3CA is involved in mutations in E542K, E545K, and H1047R positions [7,8,11], these mutations promote hyperactivation of the PI3K/AKT/mTOR pathway, which plays a key part in cancer cell growth and proliferation [3-5,7]. There are studies that show PIK3CA regulating P53 to promote tumor cell survival, proliferation, invasiveness, prognosis, and therapeutic resistance [12]. This further strengthens the role of PI3K in the progression and development of prostate cancer [9], and this pathway could be independent to the AR pathway [2].

Therefore, PI3K has been reported to be present in many cancer tissues and has been negatively associated with the poor condition and prognosis of cancer patients, because PI3K pathway is highly expressed in many cancers [4,5,7]. Accordingly, there are studies over the inhibition of the PI3K [8,13,14]. Recently we tested some compounds in breast cancer cell lines, and we proposed a cytotoxic effect due to the regulation of PIK3CA by C5 compound [8].

In this study we tested the C5 compound with prostate cancer cells (PC3) by *in vitro* cultures. The uses of PC3 cell are highlighting, because it contains key messengers that promote the viability of this cancer type

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(without AR), which make it a possible object of study, some of these messengers as FAK [15-17], P70S6K [4,5,18-20], where the PI3K pathway could has functions. As well as PI3K could regulate the apoptosis in cancer cells, thus for any development of anticancer drug is important to generate apoptosis [5,21]. There are studies related to developing PI3K inhibitors [5,7,12,14] with promising results that demonstrated that PI3K might be a good therapeutic target.

In this study, we evaluated the C5 compound that we previously reported [8], it could be interacting with PIK3CA (near of mutations to increase kinase activity), highlighting the importance of this specific site, and in this way, this compound could regulate the PI3K´s function related to prognosis of cancer [4,5,7,14], it was determined by molecular dynamics simulation, cytotoxicity, MTT, and western blot assays.

Materials and methods

Reagents

Cell culture reagents were purchased from Thermo-Fisher (Carlsbad, CA, U.S.A.), tissue culture plates and other plastic materials were obtained from Corning Inc. The following primary antibodies against p-FAK (Tyr397, sc-81493), p-P70S6K (Thr421 and Ser424, sc-377529), Caspase-3 (sc-7272), and β-actin (sc-8432) were used from Santa Cruz Biotechnology, Inc. (TX, USA), and secondary antibodies conjugated with horseradish peroxidase were used from Sigma-Aldrich (MO, USA), and C5 compound previously reported [8] was bought in Chembridge Corp. [22] (ID-Chembridge: C5:7636733, PubChem CID: 2944549). Purity of the compound reported by the manufacturer was greater than 95% checked by LC/MS, the stock solutions for compounds were prepared in Dimethyl sulfoxide (DMSO) (1-10 mM), and MTT obtained from Merck (Darmstadt, Germany).

Cell culture

The hepatocytes cells (C9) and prostate cancer cells (PC3) were obtained from American Type Culture Collection (ATCC). Cells were cultured in 100 mm Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3.7 g/L sodium bicarbonate, 5% (PC3) and 10% (C9) fetal bovine serum (FBS), and antibiotics in a humidified atmosphere containing 5% CO2 at 37 C.

Western blot analysis

Plates of six wells were used, 150,000 cells per well were seeded in 1.5 mL of supplemented DMEM medium for 48 h, incubated at 37 °C, and 5 % CO_2 allowed to reach 90 % confluence. The DMEM-supplemented medium was replaced with the DMEM experimental medium, and cells were incubated at 37 ºC for 18 h. The medium was removed, and 1 mL of experimental DMEM medium was placed under C5 compound at 50 μM of concentration. Treatments were maintained for 48 h. Later, treatments were removed, and the cell monolayer was washed once with PBS. Total protein extraction was performed using the RIPA buffer system (ChemCruz sc-24948). For protein separation, 12 % SDS-polyacrylamide gels were used for the characterization of proteins. Proteins were transferred overnight to PVDF membranes at 4 °C, 90 mA. Membranes were blocked with 5 % low-fat milk/TBST 0.1 % and later incubated with the primary antibody overnight at 4°C under gentle agitation. We evaluated these targets: p-FAK, p-P70S6K, Caspase-3, and β-actin. The β-actin was used as a loading control. Anti-mouse secondary antibodies coupled to Horseradish peroxidase from Thermo-Fisher. Chemiluminescence detection was performed using Immobilon Western kit (Millipore, MA, U.S.A.) and X-ray film for some blots, for others Bio-Rad ChemiDoc XRS+ was used, and a digital image was obtained.

Molecular dynamics simulations of C5 compound with PI3KCA

The best complex of PI3K (from the Protein Data Bank [23], PDB code 5UK8) with C5 compound was generated by molecular docking previously reported [8], and it was used for molecular dynamics simulation (MD) using Molecular Operating Environment (MOE 2024.06.01). MD was performed using Dynamics – MOE and OPLS-AA forcefield. At the beginning, in two consecutives steps of 100 ps reaching a temperature of 300 K and a pressure of 1 bar. Finally, a 10 ns of MD was carried out. Furthermore, the interactions were analyzed.

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C5 compound cytotoxicity in C9 cells determined by MTT assay

The C5 compound cytotoxic effect in C9 cells was evaluated by MTT assay, it was performed as described previously [24,25]. Briefly, C9 cells were seeded in 96-well microplates (10,000 cells per well), then were treated for 48 h at concentrations between 10 to 400 µM of C5 compound, and DMSO at a final concentration of 0.4% was used as vehicle control [24,25]. The medium was removed, and formazan crystals were dissolved in DMSO. Then, the plate was read at 570 nm by using the iMark™ Microplate Absorbance Reader (Bio-Rad, USA).

The assays were performed in triplicate in three independent experiments, and the percentage of viability was calculated according to the formula: % viability=[mean Optical Density (O.D.) treated cells×100]/(mean O.D. control cells). The concentration leading to 50 % inhibition viability (IC50) was calculated by non-linear regression analysis (percentage of viability vs. log concentration) with GraphPad Prism software ver. 8.0 software (GraphPad, CA, USA).

Statistical analysis

Data´s results were expressed as mean ± standard deviation (SD) of minimum three independent experiments. Averages and standard deviations were calculated in Excel (365 Microsoft, USA). IC50 for citotoxicity of compounds were calculated using GraphPad Prism 8 Software (La Jolla, CA, USA).

Results

Cytotoxic effect of C5 compound on PC3 cells in vitro cultures

After exposure to C5 compound for 48 h at 50 µM on PC3 cells *in vitro* cultures, a cytotoxic effect was observed, loss of monolayer, and very low viability of culture (Fig. 1). The C5 compound that we reported previously [8].

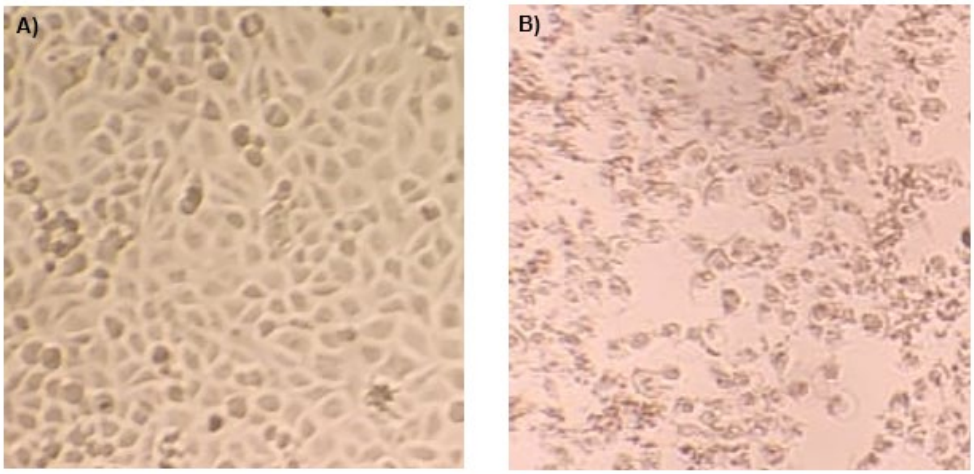

Fig. 1. PC3 cell line after 48h of incubation. A) Control with DMSO as vehicle, B) C5 compound at 50 µM.

Protein expression regulated by C5 compound on PC3 cells in vitro cultures

We determined the effect of C5 compound to 50 µM after 48 h of incubation in PC3 cells *in vitro* cultures. We determined less expression of p-FAK and p-P70S6K and higher expression of Caspase-3 (Fig. 2), these regulations were probable due to the effect of C5 compound on PI3K pathway, since these proteins are important into the proliferation signaling [15-17], and the apoptosis pathway [26].

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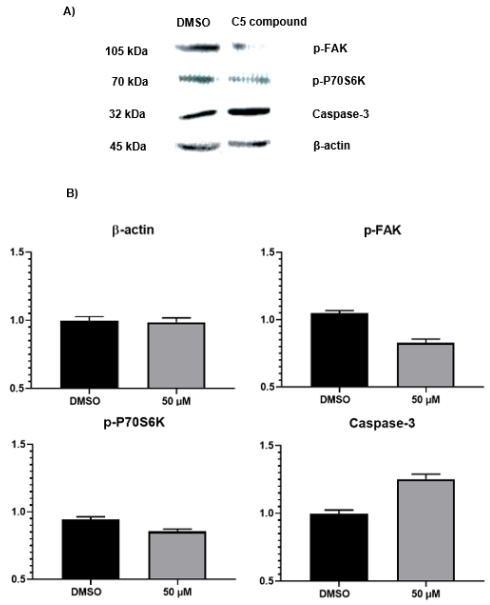

Fig. 2. Effects of C5 compound in PC3 cells. Cells were seeded with a seeding density of $1.5x10^5$ cells and were treated with the C5 compound at 50 μM for 48 h. The control cells were left untreated (DMSO). The whole cell protein lysate was prepared and 20 μg of proteins was resolved in SDS-polyacrylamide gels. **(A)** Western blot analysis of p-FAK, p-P70S6K, Caspase-3, and β-actin proteins. **(B)** Densitometry analysis of the data in Western blot analysis by ImageJ software. The values were normalized against β-actin, and they are represented as mean ± SD (n = 3).

Molecular dynamics simulation

To study in more detail the complex C5-PIK3CA mechanism, 10 ns of molecular dynamics simulation was performed. The root mean square deviation (RMSD) was analyzed, the data showed that the inhibitor formed a stable complex during simulation time, and after 10 ns were stable the main interactions, and notably interactions with Glu365 and Pro539 (Fig. 3). The overall RMSD after 10 ns was 2.60 A, and specifically in the amino acids in the active region the RMSD were between 0.69 to 1.24 A (Table 1). These interactions are close to the activation region, mainly in position 542 in PIK3CA (Fig. 4), and it could block exposure to the mutations reported for PIK3CA enzyme to increase kinase activity and their functions in cancer [3-5,7,8,11].

Table 1. Main interactions and RMSD of C5 with PIK3CA between 0 – 10 ns.

Time	Ligand	Receptor (Residues in PIK3CA)	Interaction	Distance	RMSD (A)
0 ns	N	GLU365	H-donor	2.74	-
	N	PRO539	H-donor	2.82	-
	6-ring	TYR361	pi-H	4.01	
5 ns	N	PRO539	H-donor	3.42	0.93
	O	ASN605	H-acceptor	3.02	0.71
	C	PHE1016	H-pi	3.79	1.24

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Time	Ligand	Receptor (Residues in PIK3CA)	Interaction	Distance	RMSD (A)
10 ns	N	GLU365	H-donor	2.63	1.15
	N	PRO539	H-donor	2.95	0.69
	C	GLU365	H-donor	3.51	1.15

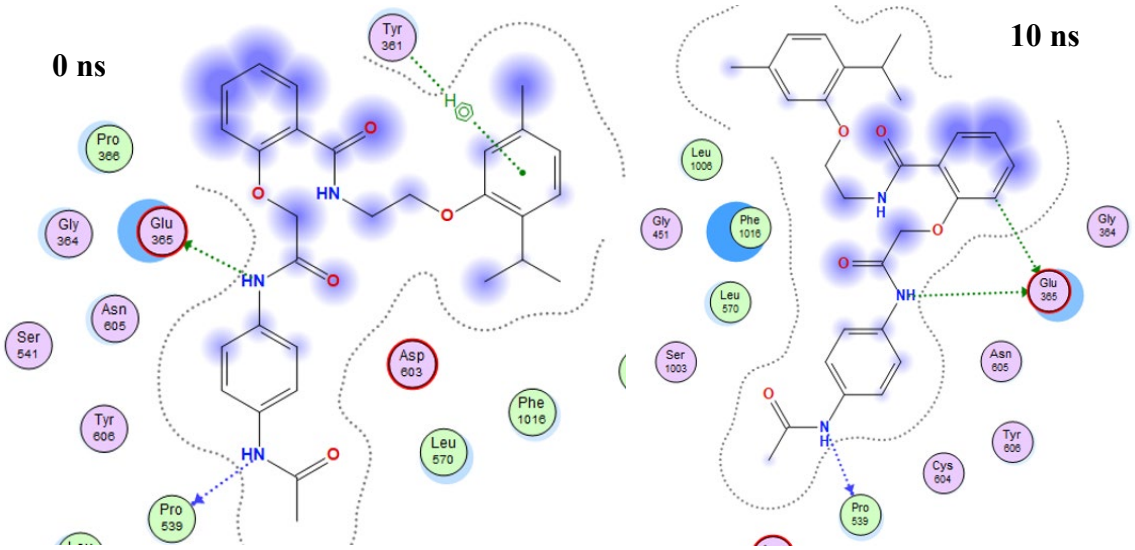

Fig. 3. Interactions at 0 and 10 ns from molecular dynamics simulation between C5 compound and PIK3CA.

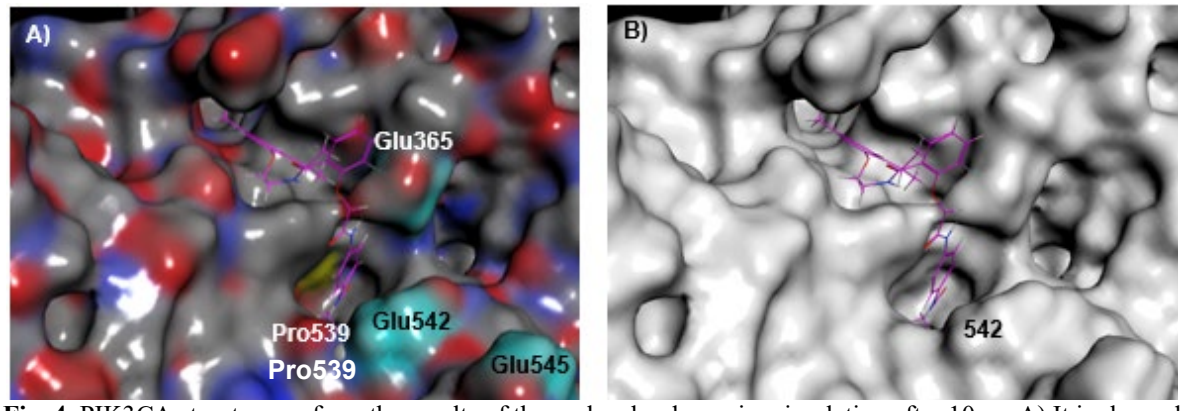

Fig. 4. PIK3CA structure surface, the results of the molecular dynamics simulation after 10 ns. A) It is showed the main amino acids the interactions between the C5 compound. B) It is showed the region of activation region (white) and the colocation of C5 compound (pink), for blocking the access to activation region near to amino acid in position 542.

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C5 compound cytotoxicity in C9 cells determined by MTT assay

We tested the C5 compound on hepatocytes cells (C9, a cell line normal without relation to characteristics of cancer) *in vitro* cultures between 10 to 400 μM of concentration, and all results were favorable; C5 compound without cytotoxic effect on C9 cells *in vitro* cultures incubation for 48 h. In addition, C5 compound cytotoxic *in vitro* results are related to *in silico* results previously reported [8].

Discussion

In this study we evaluated one compound previously reported [8], to develop a specific pharmacological treatment to regulate the PI3K pathway focusing mainly on cell proliferation and migration mechanism [3-5,7,14], due to PIK3CA activity is related to promoting invasion and tumorigenesis by several mechanisms. PIK3CA is a key protein involved in cellular signaling in mechanisms of cell proliferation, survival and apoptosis. PIK3CA is a subunit of PI3K, that when it is activated, it induces the production of PIP3 which activates the AKT [3,7]. Once activated, AKT activates mTOR, activating P70S6K and cellular proliferation, by promoting cell growth and division [18,19]. Another mechanism in which PIK3CA is involved, it is in the inhibition of pro-apoptotic factors in the activation of AKT, promoting cell survival, which reduces the activation of caspase-3 and inhibiting the apoptosis process [26]. As mentioned before, oncogenic mutations in E542K, E545K, and H1047R positions in PIK3CA promote hyperactivation of the PI3K/AKT/mTOR pathway, playing an important role in tumor progression [7,8,11]. All these mechanisms become PIK3CA an object of study for drug development in anticancer treatments.

There are studies to develop specific inhibitors involved in the PI3K/AKT/mTOR pathway [7,8]; thus, our study focuses on determining the effect of C5 compound over PI3K pathway in prostate cancer cells without AR depending (PC3 cell). In this kind of prostate cancer are reported important messengers to regulate the viability of this cancer type, some of these messengers are p-FAK and p-P70S6K [15,16,19]. In this way, our results of western blot assays showed a possible regulation in the PI3K pathway for proliferation and apoptosis; since C5 compound generated decreasing in the signal for p-P70S6K and p-FAK, and both proteins are related to the regulation of pathogenesis and progression of cancer [15,16,18,19], and in this way, to decrease the viability of prostate cancer cells (Fig. 2). On the other hand, it was determined the increasing expression of Caspase-3 after 48 h (Fig. 2), it is important, due to there are studies that show the relevance to generate apoptosis by the increasing of caspases [27-30], and our results showed changes morphological and loss of monolayer in the *in vitro* assays, that are relating to the cytotoxic effect of C5 compound on PC3 cells (Fig. 1), and probably to promoting the apoptosis process [31-33]. Therefore, we propose that C5 compound has an effect on PI3K pathway, decreasing this signaling (over FAK and P70S6K), in this way it is decreasing the viability of the culture, and continues with apoptosis.

To justify the cytotoxic and apoptosis effect by *in silico* assays, first, results of molecular docking reported [8] showed that the C5 compound could have interaction with PIK3CA in a region considered as potential therapeutic target (activation region) [3-5,7,8]. These interactions could regulate a less signaling of PI3K pathway to generate less proliferation/migration [3-5,14], besides the results of the molecular dynamics simulation showed that the interactions of the C5 compound in the activation region probably are stable (Fig. 3 and 4), and we describe the main interactions of C5 compound with PIK3CA (Table 1), the interactions are very similar either in the molecular docking and in the molecular dynamics simulation (Glu365 and Pro539), in the PIK3CA´s activation region (Table 1). Therefore, these interactions could decrease some PIK3CA´s functions, where studies reported this region to generate the inhibition of this protein (mainly in position 542), that could block exposure to the mutations reported for PIK3CA enzyme to increase kinase activity and their functions in cancer (E542K and E545K) [3-5,7,8,11].

It is important to mention the relevance to regulate the migration and proliferation in prostate cancer, due to AR or PI3K pathway. Androgens, specifically testosterone, and dihydrotestosterone (DHT), are the major ligands for AR. There are multiple mechanisms of resistance contribute to the progression of hormone-sensitive prostate cancer to castration-resistant prostate cancer (CRPC) [2,34-36], where there are approved therapies for CRPC (docetaxel and cabazitaxel), even agents targeting the resistance pathways leading to CRPC

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(enzalutamide and abiraterone) [2]. Despite these treatments, other treatments against new targets in PCa are needed, and one of them is into the PI3K/AKT pathway.

Regarding other PI3K inhibitors, specifically against PIK3CA there are on different subunits into this enzyme. Particularly, against subunit p110-alpha, an inhibitor studied is Alpalesib [37,38], that it had been tested on different cancer cell lines, and studied its effect on PI3K pathway. As well as Alpalesib, the C5 compound could interact around the subunit p110-alpha and it had been tested against MDA-MB-231 and MCF7 cell in vitro cultures [8]. In this way, other potential treatments or adjuvants could be added in the future to reduce cell migration and proliferation, and these could be combined with other treatments with different mechanisms of action (AR, ADT or CRPC) against PCa.

Therefore, the C5 compound could generate a down regulation of the PI3K pathway, in addition the C5 compound is potentially secure to use in humans, due to in this study we wide the cytotoxic assays with normal cells (hepatocytes), and these results were favorable. With these results, the C5 compound has more results to continue the development of a new anticancer drug.

Conclusions

We propose the C5 compound to develop a new anticancer drug, that could be used against prostate cancer depending on PI3K pathway, due to the findings shown in this study. We describe that the C5 compound could have a regulation over PI3K pathway, by the regulation of the expression proteins related to proliferation cancer cells (p-FAK and p-P70S6K). Finally, the C5 compound could have specific interactions with PIK3CA (in the region p110-alpha, nearly to activation region), and probably safe for humans. We promote more assays to continue this development of new anticancer drug.

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