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# Effect of econazole on $Ca^{2+}$ signaling in human colorectal adenocarcinoma cells

## [İnsan kolorektal adenokarsinom hücrelerindeki $Ca^{2+}$ sinyal iletimi üzerine ekonazolün etkisi]*

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**ABSTRACT**

**Introduction:** Econazole, an azole compound widely used as an antifungal drug, is currently investigated for additional therapeutic effects. In fact, the antitumoral properties of econazole have been recently demonstrated, both in vivo and at the cellular level. However, the precise mechanism of action behind its effects is still unclear.

**Aim:** To examine the effect of econazole on intracellular $Ca^{2+}$ signaling pathways in a human adenocarcinoma cell line.

**Methods:** $[Ca^{2+}]_i$ was measured in Fura 2-loaded HT29 cells by fluorescence. Fluorescence was recorded from 1ml aliquots of magnetically stirred HT29 cells using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 510 nm.

**Results:** We have demonstrated that econazole has a dual effect on $Ca^{2+}$ homeostasis. It caused a dose-dependent increase in $[Ca^{2+}]_i$. Both mobilization from thapsigargin-sensitive stores and extracellular $Ca^{2+}$ influx contributed to the effect. A phospholipase C-dependent pathway proved to be activated by econazole. In addition, econazole greatly inhibited a physiological activation of store-operated $Ca^{2+}$ entry.

**Conclusion:** This study provides further molecular mechanism of econazole suggesting that this drug may modify the physiology of human adenocarcinoma HT29 cell line.

**Key Words:** Econazole, $Ca^{2+}$ signaling, HT29 cells.

**Conflict of Interest:** The authors declare that there is no conflict of interest.

**ÖZET**

**Giriş:** Geniş kullanım alanına sahip, antifungal etkili Ekonazol bir azol bileşiğidir ve şimdilerde farklı terapotik etkileri çalışılmaktadır. Son zamanlarda ekonazolün antitümoral etkileri hem in vivo hem de hücresel düzeyin de gösterilmiştir. Bununla birlikte, etki mekanizması halen kesin belli değildir.

**Amaç:** Çalışmanın amacı İnsan adenokarsinom hücre kültürlerinde Ekonazolün hücre içi $Ca^{2+}$ sinyal iletimi yollarındaki etkisinin belirlenmesidir.

**Metot:** $Ca^{2+}$ konsantrasyonu Fura 2-yüklenmiş HT29 hücrelerinde floresan işaretlemeyle ölçülmüştür. Cary Eclipse spektrofotometre (Varian Ltd., Madrid, Spain) kullanılarak, 1 ml'lik karıştırılmış HT29 hücrelerinde, 340 ve 380 nm eksitasyon dalga boylarında ve 510 nm emisyonda fluoresans ölçülmüştür.

**Bulgular:** Ekonazolün $Ca^{2+}$ homeostazı üzerine dual etkisi olduğu gösterilmiştir. Bu etki doza bağımlı $Ca^{2+}$ konsatrasyonundaki artış sonucunda görülmüştür. Hem Tapsigargin-duyarlı depolardan hem de hücredışından $Ca^{2+}$ girişi etkiye katkıda bulunmuştur. Fosfolipaz C-bağımlı bir yolağın ekonazol tarafından aktive edildiği ispatlanmıştır. Ek olarak, ekonazolün $Ca^{2+}$'un depolanmak üzere fizyolojik aktivasyonunu yüksek oranda inhibe ettiği gösterilmiştir.

**Sonuç:** Bu çalışma, Ekonazolün insan adenokarsinom HT29 hücre kültürlerinin fizyolojisini çeşitli moleküler mekanizmalarla etkilediğini göstermiştir.

**Anahtar Kelimeler:** Ekonazol, $Ca^{2+}$ sinyal iletimi, HT29 hücre kültürü.

**Çıkar Çatışması:** Yazarların çıkar çatışması yoktur.

## Introduction

The discovery of the antifungal activity of azole compounds represented an important therapeutic advance [1-5]. Within them, Econazole, widely used as an antifungal drug [6], is currently further investigated for additional therapeutic effects.

In fact, both econazole and ketoconazole (1 nM - 1 μM) decreased human breast cancer MCF-7 cell proliferation in a time and dose-dependent manner [7]. In addition, tumor growth was slightly delayed in liposomal econazole-treated mice [8]. Moreover, significant anti-tumorigenesis effect was demonstrated in vivo by treating nude mice bearing COLO 205 tumor xenograpfts with econazole 50 mg/kg intraperitoneally [9] and the same researchers also reported that lower doses of econazole (5 - 20 μM) arrested human colon cancer cells at G0/G1 phase of the cell cycle.

Different molecular mechanisms stay behind the clinical effects of drugs. It is well established that $Ca^{2+}$ is among the major intracellular factors involved in the signaling transduction pathways evoking cell growth and proliferation, and other key processes such as gene expression [10,11]. When calcium signaling is stimulated in a cell, $Ca^{2+}$ enters the cytoplasm from one of two general sources: it is released from intracellular stores, or it enters the cell across the plasma membrane. Both processes often occur either simultaneously or sequentially [12]. Within $Ca^{2+}$ mechanisms contributing to the increases in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), particular attention has been paid to the role of $Ca^{2+}$ entry through store-operated channels (SOC) [13]. Thus, store-operated $Ca^{2+}$ entry (SOCE), a refilling process triggered by depletion of $Ca^{2+}$ stores, has been involved in cell signaling occurring in non-excitable cells to evoke different cell processes including gene regulation and cell growth [14]. Entry in cell cycle is preceded by SOC activation [15] and SOCE inhibition by different means abolishes tumor cell proliferation [16-19]. In addition, alterations in intracellular $Ca^{2+}$ homeostasis are commonly observed during apoptosis [20,21] and it has been demonstrated that the depletion of the endoplasmic reticulum $Ca^{2+}$ stores can directly induce apoptosis [22].

Although several reports have already suggested the antitumor effect of econazole, its molecular mechanism of action is still unclear. Being calcium signaling an essential tool for the development of different cellular responses as cell proliferation or apoptosis [23-26] and being colorectal cancer one of the commonest tumors in the Westernized world [27], the present study aims to investigate the role of econazole on $Ca^{2+}$ signaling in a human adenocarcinoma cell line. Previous reports showed the effect of econazole on $Ca^{2+}$ movements in different cell lines [28-30], but our paper is the first to investigate the role of econazole on $Ca^{2+}$ signaling in HT29 cells.

## Materials and Methods

HT29 cells were kindly donated by C. Villalobos (CSIC, Valladolid). Fura-2/AM was purchased from Fluka. Carbachol, econazole, PMA, propanolol, staurosporine and thapsigargin from Sigma. U73122 and nifedipine from Calbiochem. Reagents for cell culture were purchased from GIBCO.

The human colorectal adenocarcinoma cell line HT29 was maintained in standard DMEM, (complemented with 10% fetal bovine serum (FBS), 1% peniciline/ streptomicine and 1% glutamine) and kept in an incubator (37ºC, 10% $CO_2$ humidified atmosphere).

Cells were propagated in 75 $cm^2$ culture flasks, detached by trypsin treatment, washed in culture medium and loaded with 4 μM of the fluorescence dye Fura-2/ AM (a ratiometricfluorescent dye which binds to free intracellularcalcium) for 60 min at room temperature in an standard solution ($Ca^{2+}$-standard medium containing (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; HEPES, 10 (pH 7.4)). Fluorescence was recorded from 1ml aliquots of magnetically stirred HT29 cells ($1.5x10^6$ C/mL) at 37ºC using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. When experiments were performed in a $Ca^{2+}$-free medium, extracellular $Ca^{2+}$ was chelated with 5 mM EGTA-Tris addition. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 fluorescence ratio (340/380). The $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz et al. [31]. Increases in $[Ca2+]_i$ were expressed as nM (mean ± SD).

### *$Mn^{2+}$ measurements*

$Mn^{2+}$ measurements were performed as previously described by our group in HT29 and other cell types [32,33]. Briefly, $Mn^{2+}$ uptake was monitored as a rate of quenching of Fura-2 fluorescence measured at the $Ca^{2+}$-insensitive wavelengths (excitation 360 nm and emission 510 nm). The rate of the fluorescence decrease provides a relative measure of the divalent cations permeability.

Analysis of statistical significance was performed using Statgraphics Centurion XVI. The data are represented as mean ± SD for each group. One-way ANOVA was used to analyse the statistical significance between mean values followed by a least-significant difference (LSD) test. $p < 0.05$ was taken as the minimum level of significance.

## Results

Econazole increased $[Ca^{2+}]_i$ in a concentration-dependent manner in the presence of extracellular $Ca^{2+}$ in HT29 cells. Fig. 1 shows the recording of 4 different concentrations (μM) 0.1, 1, 2.5, 10 of econazole. Increasing the concentration above 1 μM clearly induce an $[Ca^{2+}]_i$ increase, reaching the maximum net value at the

higher concentrations. When 20 μM was tested no bigger $[Ca^{2+}]_i$ increase was observed (data not shown).

We conducted experiments in the absence of extracellular $Ca^{2+}$ to assess whether an influx of extracellular $Ca^{2+}$ or mobilization of $Ca^{2+}$ from the intracellular pools may contribute to econazole-induced $[Ca^{2+}]_i$ rises. Extracellular $Ca^{2+}$ was chelated by addition of EGTA-Tris to the medium. Assuming no extracellular $Ca^{2+}$ was allowed to enter, any increase in $[Ca^{2+}]_i$ must arise from $Ca^{2+}$ release from the stores. Statistical differences ($p < 0.05$) were found when comparing econazole maximum peak in both media. Fig. 2 shows that econazole response in a $Ca^{2+}$-free medium is greatly curtailed if compared with the one observed in a $Ca^{2+}$-containing medium. Thus, both extracellular $Ca^{2+}$ influx and intracellular $Ca^{2+}$ release were induced by econazole, with the former one taking a dominant role.

We performed experiments to further investigate the regulation of econazole-induced $Ca^{2+}$ signal. Nifedipine (10 μM), staurosporine (STA) (1 μM) and propanolol (150 μM) did not alter 10 μM econazole-induced $Ca^{2+}$ movements in a $Ca^{2+}$-containing medium. Nevertheless, $Ni^{2+}$ (1 mM) inhibited econazole-induced $[Ca^{2+}]_i$ rise in HT29 cells. Statistical differences ($p < 0.05$) were found when comparing econazole response in control cells and $Ni^{2+}$-treated cells (Fig. 3).

In order to elucidate the origin of the calcium recruited by econazole, we used thapsigargin (Tg). Tg without acting on $IP_3$ receptors, increases cytosolic $Ca^{2+}$ by inhibiting the $Ca^{2+}$-ATPase present on the endoplasmic reticulum [34]. Application of 400 nM Tg, in a $Ca^{2+}$-free medium, caused an $[Ca^{2+}]_i$ increase that comprised an initial rise and a gradual decay towards baseline; after depleting the endoplasmic reticulum $Ca^{2+}$ store with Tg, addition of 10 μM econazole did not induce any $Ca^{2+}$ increase, thus suggesting that the internal $Ca^{2+}$ source for the econazole response is the Tg-sensitive endoplasmic reticulum store (Fig. 4). Moreover, addition of 400 nM Tg after treating cells with econazole, also failed to induce a $[Ca^{2+}]_i$ rise (data not shown). This signal most likely reflected endoplasmic reticulum $Ca^{2+}$ release induced by this drug.

Once shown that econazole recruited $Ca^{2+}$ from intracellular stores, we aimed to evaluate whether econazole evoked increases in $[Ca^{2+}]_i$ via the production of inositol 1,4,5-triphosphate ($IP_3$). We used U73122, a PLC inhibitor, to block formation of $IP_3$ [35-37]. In cells treated with U73122 econazole-induced $[Ca^{2+}]_i$ was significantly curtailed (Fig. 5).

Econazole is generally recognized as an inhibitor of SOCE in a number of cells [38-40], so we asked next whether it was able to inhibit SOCE in human colorectal adenocarcinoma cells.

Carbachol (Cch) activates $M_3$ muscarinic receptors in HT29 cells provoking a biphasic $[Ca^{2+}]_i$ increase, a peak due to the transient release from intracellular stores followed by a plateau due to sustained $Ca^{2+}$ entry via store-operated channels (SOC) [41]. Thus, we were tempted to assess whether econazole inhibited SOCE induced by a physiological stimulation with Cch in HT29 cells. Econazole inhibited $[Ca^{2+}]_i$ increase induced by carbachol in a dose-dependent manner. The maximum effect was observed when 10 μM econazole was tested (Fig. 6). Both 0.1 and 1 μM of econazole failed to induce any inhibitory effect (data not shown).

We developed experiments with $Mn^{2+}$, used as a tracer for $Ca^{2+}$ entry [42], in order to establish whether the econazole-induced decrease in $[Ca^{2+}]_i$ was actually due to an inhibition of $Ca^{2+}$ influx. We used Tg to induce SOCE. Fig. 7 shows how econazole inhibited $Mn^{2+}$ influx in cells treated with Tg.

Since it has been previously demonstrated the SOCE-inhibitory properties of phorbol 12-myristate 13-acetate (PMA), in several cell types [43,44], we tested the effect of such a phorbol in HT29 cells. Fig. 8 shows that activation of protein kinase C (PKC) inhibited $[Ca^{2+}]_i$ rise induced by Cch in HT29 cells. However, inhibition of PKC with STA, did not affect the change in $[Ca^{2+}]_i$ evoked by econazole. Thus, the inhibition of the Cch-evoked $[Ca^{2+}]_i$ transient by econazole was apparently PKC-independent.

## Discussion

The main finding of the present report is that econazole has several effects on $Ca^{2+}$ movements in HT29 cells. Both extracellular $Ca^{2+}$ entry and intracellular $Ca^{2+}$ release from the stores are affected by econazole.

Our data suggest that econazole induced a dose-dependent increase on $[Ca^{2+}]_i$. Concentrations above 1 μM greatly induced an $[Ca^{2+}]_i$ rise, giving the maximum peak the higher concentrations tested (10-20 μM). Similar results were previously reported in different cell lines [28-30,45]. After 10 μM econazole stimulation, there was a pronounce initial peak followed by a rapid decline to near pre-stimulation level of $[Ca^{2+}]_i$. Both intracellular $Ca^{2+}$ release from the stores and extracellular $Ca^{2+}$ influx contributed to the $[Ca^{2+}]_i$ rise, which is in agreement with results previously stated [28-30]. Calcium entry was greatly curtailed in cells treated with $Ni^{2+}$, but it remains to be uncertain the type of channels implicated, as nifedipine, an L-type channels antagonist, failed to inhibit the entry, in clear connection with previous reports [28,29]. Moreover, a phospholipase D (PLD) pathway did not seem to be implicated in econazole response, as cells treated with propanolol failed to abolish the econazole response.

Concerning the stores implicated in the econazole-induced $Ca^{2+}$ release, thapsigargin-sensitive stores proved to be econazole's target, as demonstrated for different cell lines [28,29]. U73122, a pharmacological inhibitor of PLC, significantly abolished the econazole-induced rise in $[Ca^{2+}]_i$. Activation of PLC produces

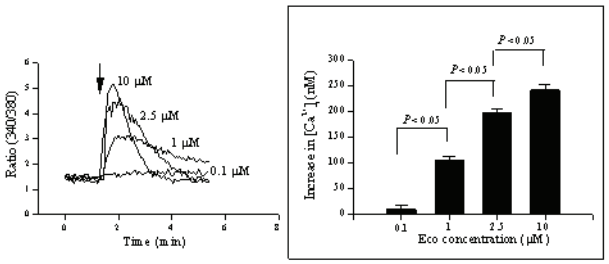


**Figure 1.** Effect of econazole (Eco) on $[Ca^{2+}]_i$ in HT29 cells. The experiments were performed in a $Ca^{2+}$-containing medium (1 mM). Eco (0.1-10 µM) was added to the cells at the time indicated by the arrow. The curves, represented as Ratio (340/380), show the single traces of observations which were reproduced several times independently (n≥6). Data in histograms represented as mean (SD) values of the increases in $[Ca^{2+}]_i$ (nM) were analyzed by employing LSD test of significance. The calibration of the experiment was performed as described in Material and Methods.

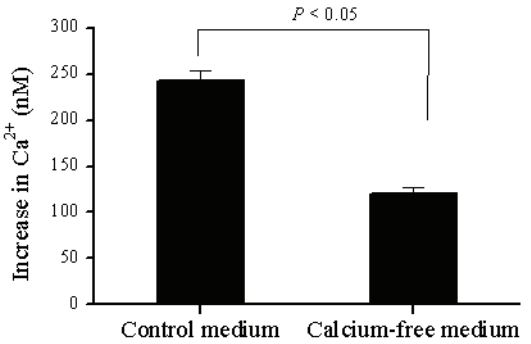


**Figure 2.** Effect of 10 µM econazole on $[Ca^{2+}]_i$ in a $Ca^{2+}$-free medium in HT29 cells. Data in histograms are represented as mean (SD) of three independent experiments and analyzed by employing LSD test of significance ($p < 0.05$). A comparison of the effects of econazole in a $Ca^{2+}$-free medium and in a "control medium" (containing 1 mM $Ca^{2+}$) is shown.

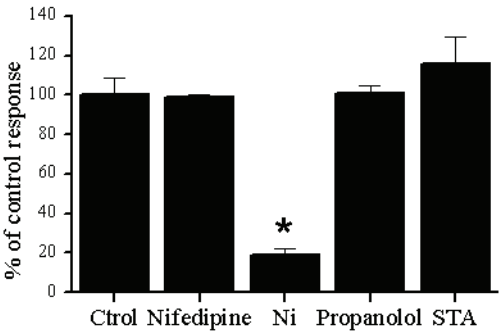


**Figure 3.** Effect of modulators of protein kinases C (PKC) and blockers of $Ca^{2+}$ channels on econazole-induced $[Ca^{2+}]_i$ rise. Experiments were performed in $Ca^{2+}$ containing medium. Nifedipine (10 µM), $Ni^{2+}$ (1 mM), propanolol (150 µM) and staurosporine (STA) (1 µM) were applied 10 minutes before addition of 10 µM econazole. Data in histograms are represented as mean (SD) of three independent experiments and analyzed by employing LSD test of significance (*$p$< 0.05).

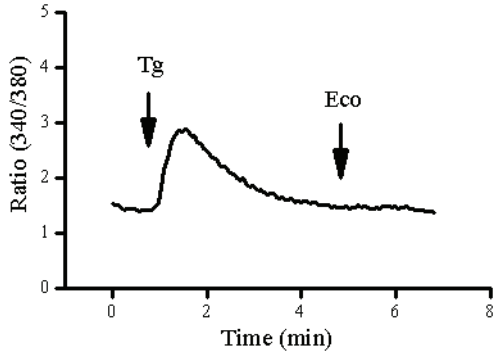


**Figure 4.** Effect of econazole (Eco) on thapsigargin (Tg)-sensitive stores. The experiments were performed in a $Ca^{2+}$-free medium. 400 nM Tg and 10 µM Eco were added at the time indicated by the arrows. The curves, represented as Ratio (340/380), show the single traces of observations which were reproduced several times independently (n≥6).

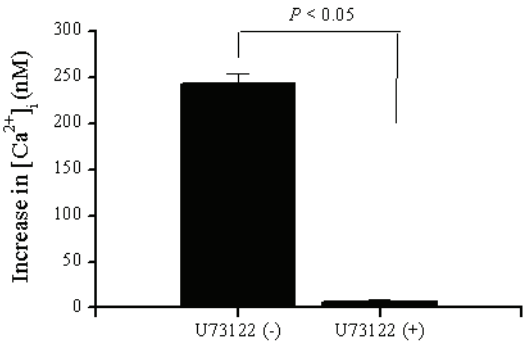


**Figure 5.** Effect of econazole (Eco) on the increases in $[Ca^{2+}]_i$ in U73122 treated cells. Before adding econazole (10 µM), cells were incubated during 5 minutes with 3 µM U73122. U73122(-) represents non-treated cells. U73122(+) represents U73122-treated cells. Data in histograms represented as mean (SD) values of the increases in $[Ca^{2+}]_i$ (nM) were analyzed by employing LSD test of significance. The calibration of the experiment was performed as described in Material and Methods.

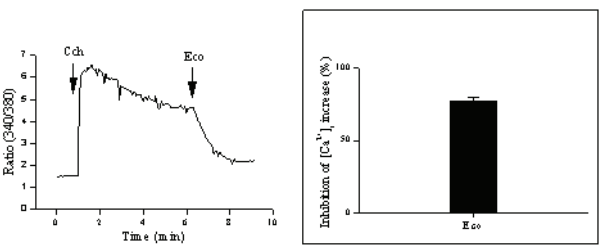


**Figure 6.** Effect of econazole (Eco) on carbachol (Cch)-induced SOCE. Experiments were performed in a $Ca^{2+}$-containing medium. 100 µM Cch and 10 µM Eco were added when indicated by the arrows. The curves, represented as Ratio (340/380), show the single traces of observations which were reproduced several times independently (n≥6). Data in histograms represented as mean (SD) values of the inhibition of the increases in $[Ca^{2+}]_i$ (%) were analyzed by employing LSD test of significance. The calibration of the experiment was performed as described in Material and Methods.

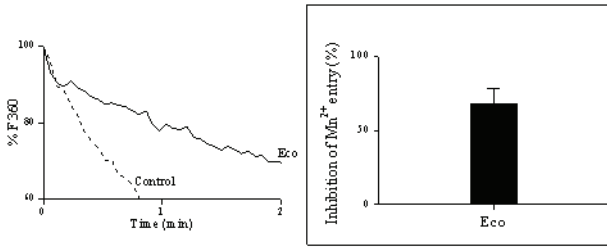


**Figure 7.** Effect of econazole (Eco) on $Mn^{2+}$ entry in Carbachol (Cch)-treated cells. "Control line" represents cells treated with 100 µM Cch. Eco line represents cells in which SOCE has been induced by Cch, and immediately after Eco (10 µM) was added to the cuvette. For $Mn^{2+}$ entry measurements, 1 mM $MnCl_2$ was added and the decrease of the fluorescence excited at 360 nm was followed. Readings have been normalized to 100% at the time of $Mn^{2+}$ addition. The curves show the single traces of observations which were reproduced several times independently (n≥6). Data in histograms represented as mean (SD) values of inhibition of $Mn^{2+}$ entry (%), were analyzed by employing LSD test of significance.

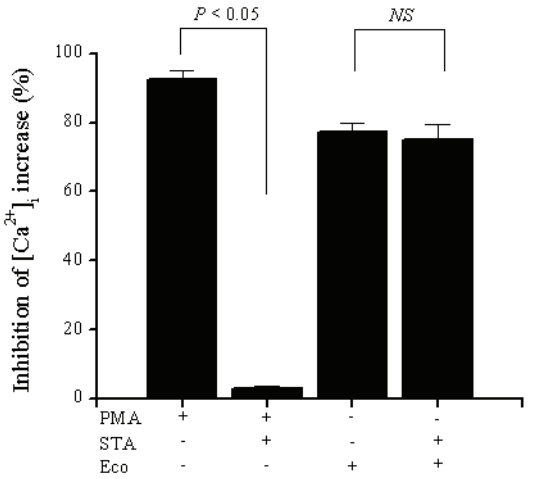


**Figure 8.** Comparison of the effects of staurosporine (STA) on the inhibition of SOCE induced by either PMA or econazole (Eco) in HT29 cells. Carbachol (Cch) (100 µM) was used to induce SOCE in a $Ca^{2+}$-containing medium. PMA and Eco were added at 0.1 µM and 10 µM, respectively. Data in histograms, represented as mean (SD) values of inhibition of the increases in $[Ca^{2+}]_i$ (%), were analyzed by employing LSD test of significance (n≥6). NS, no significant differences.

$IP_3$ and diaylglycerol, which activates PKC. However, econazole-induced $Ca^{2+}$ increase was not inhibited by treating the cells with staurosporine, a PKC inhibitor, thus suggesting that the $[Ca^{2+}]_i$ rise observed after treating the cells with econazole was apparently not dependent on PKC activation.

Because $Ca^{2+}$ overloading is known to initiate processes leading to cell death [46], our results may suggest a molecular mechanism of action for the reported apoptotic properties of econazole [9].

Econazole has been shown to inhibit store-operated $Ca^{2+}$ entry in several cell types, so we explored the effect of econazole in HT29 cells. In fact, our results showed that econazole greatly inhibited SOCE induced by physiological stimulation with carbachol. In agreement with previous studies for several cell lines [47,48], we have demonstrated that activation of PKC, using phorbol esters, produces inhibition of agonist-induced $[Ca^{2+}]_i$ rise in HT29 cells. However, such a pathway was not involved in the inhibitor effect induced by econazole, as cells treated with staurosporine, failed to prevent such an inhibition. The lack of further evidence let us support those studies suggesting a link between a wide range of cytochrome P450 inhibitors and their role as blockers of SOCE [49,50]. However, other studies disagree on this hypothesis [38,40,51] reporting that other imidazole compounds failed to have such an effect. Moreover, previous reports [52] showed that adding BSA removed the inhibitory effect of econazole in Ehrlich ascites tumour cells. In this context, it has been also demostrated that econazole did not inhibit $I_{CRAC}$ ($Ca^{2+}$ release-activated $Ca^{2+}$ channels) when injected intracellularly but did so when presented externally by patch clamp pipette [53]. But whatever the mechanism of $Ca^{2+}$ entry inhibition is, inhibition of SOCE has been shown to block cell proliferation in tumour cells [17,18]. If SOC-E is essential for cell proliferation then it follows that drugs interfering with such a pathway may prevent cell proliferation via SOC-inactivation. Núñez et al. reported SOCE-inhibition as a novel mechanism that may contribute to explain the reported anti-proliferative and anti-tumoral actions of aspirin and dietary salicylates, which is also in agreement with several groups reporting that the inhibition of SOCE contributes to the anti-proliferative effect of drugs in human colon cancer cells [17-19]. In this sense, our results could be even connected with those previously stated demonstrating that low doses of econazole induce growth inhibition in colon cancer cells trough G0/G1 cell cycle arrest [9].

Econazole is widely used in different clinical preparations, but its effects on HT29 cells had not been previously examined. Our results show that econazole significantly alters $Ca^{2+}$ movements in these cells. Apart from its role as a SOCE blocker, it induces dose-dependent increases in $[Ca^{2+}]_i$ both by releasing $Ca^{2+}$ from the thapsigargin-sentitive stores and by inducing extracellular calcium influx. As $Ca^{2+}$ entry into the cytoplasm influences the expression and activity of intracellular kinases and gene expression [23-26], we conclude that econazole may modify the physiology of human adenocarcinoma HT29 cells.

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**Conflict of Interest:** The authors declare that there is no conflict of interest.

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