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Original Article

Cytotoxicity of capsaicin and its analogs *in vitro*

Citotoxicidade da capsaicina e seus análogos *in vitro*

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Abstract

Capsaicin (CAP) is the main compound responsible for the spicy flavor of *Capsicum* plants. However, its application can be inhibited due to its pungency and toxicity. This study aimed to evaluate and compare the cytotoxic effect of CAP and its analogs N-benzylbutanamide (AN1), N-(3-methoxybenzyl) butanamide (AN2), N-(4-hydroxy-3-methoxybenzyl) butanamide (AN3), N-(4-hydroxy-3-methoxybenzyl) hexanamide (AN4) and N-(4-hydroxy-3-methoxybenzyl) tetradecanamide (AN5) on the hepatoma cells of *Rattus norvegicus* using the MTT test. The results showed cytotoxicity of CAP at concentrations of 100, 150, 175, and 200 µM (24 hours), AN1 at 150 and 175 µM (48 hours), AN2 at 50 µM (24 hours) and 10, 25, 50, and 75 µM (48 hours), AN4 at 175 µM (24 hours), and AN5 at 50 µM (48 hours). Removing the hydroxyl radical from the vanillyl group of capsaicin, together with reducing the acyl chain to 3 carbons, which is the case of AN2, resulted in the best biological activity. Increasing the carbon chain in the acyl group of the capsaicin molecule, which is the case of AN5, also showed evident cytotoxic effects. The present study proves that the chemical modifications of capsaicin changed its biological activity.

Keywords: biological activity, *Capsicum*, MTT assay, pepper, pungency.

Resumo

A capsaicina (CAP) é o principal composto responsável pelo sabor picante das plantas de *Capsicum*. No entanto sua aplicação pode ser inibida devido à sua pungência e toxicidade. O objetivo do presente estudo foi avaliar e comparar o efeito citotóxico do CAP e seus análogos N-benzilbutanamida (AN1), N-(3-metoxibenzil)butanamida (AN2), N -(4-hidroxi-3-metoxibenzil)butanamida (AN3), N-(4-hidroxi-3-metoxibenzil) hexanamida (AN4) e N-(4-hidroxi-3-metoxibenzil) tetradecanamida (AN5) em células do hepatoma de *Rattus norvegicus* pelo teste do MTT. Os resultados mostraram citotoxicidade da CAP em concentrações de 100, 150, 175 e 200 µM (24 horas), AN1 em 150 e 175 µM (48 horas), AN2 em 50 µM (24 horas) e 10, 25, 50 e 75 µM (48 horas), AN4 em 175 µM (24 horas) e AN5 em 50 µM (48 horas). A remoção do radical hidroxila do grupo vanilil da capsaicina, juntamente com a redução da cadeia acila para 3 carbonos, caso do AN2, foi o que resultou na melhor atividade biológica. O aumento da cadeia carbônica no grupo acil da molécula de capsaicina, caso da AN5, também demonstrou efeitos citotóxicos evidentes. O presente estudo comprova que as modificações químicas da capsaicina alteraram sua atividade biológica.

Palavras-chave: atividade biológica, *Capsicum*, ensaio do MTT, pimenta, pungência.

1. Introduction

The pungency of chili peppers (genus *Capsicum*) is formed from a mixture of substances called capsaicinoids, of which the most abundant is capsaicin (8-methyl-N-vanylyl-6-nonenamide), followed by dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin (Zhigila et al., 2014; Lewinska et al., 2015). Capsaicin (CAP), besides giving a spicy flavor to chili peppers, also has a high therapeutic potential (Freitas et al., 2018), with anti-inflammatory, antioxidant, anti-obesity, analgesic, antiparasitic, immunomodulatory, and anti-tumor properties, in addition to being efficient in the treatment of arthritis-related pain, cystitis, and diabetic neuropathy (Chapa-Oliver and Mejía-Teniente, 2016; Bogusz Junior et al., 2018; Xiang et al., 2021).

On the other hand, due to its pungency, capsaicin promotes irritability in its contact with the skin, eyes, and mucosa (Iida et al., 2003). Recent research has shown that a person may feel side effects such as heartburn,

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gastrointestinal pain, and diarrhea (Xiang et al., 2021). This burning characteristic is closely linked to the length of the acyl chain and/or the substituents on the vanillyl ring (Castillo et al., 2007).

There is great interest in natural products being modified synthetically by chemical means to improve their biological profile and, thus, reduce pharmacokinetic problems and improve biopharmaceutical properties (Jana et al., 2010; Stuurman et al., 2013). According to Bohlin et al. (2010), natural products may not be active themselves, but their analogs may have interesting bioactive molecules for biological studies.

A common test to determine the cytotoxicity of several drugs at different concentrations is the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Cordeiro et al., 2021; Kamal et al., 2022; Vendruscolo et al., 2022). The MTT test is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity (Van Meerloo et al., 2011). The objective of the present study was to evaluate and compare the cytotoxic effect of CAP and its less pungent analogs, viz. n-benzylbutanamide (AN1), N-(3-methoxybenzyl) butanamide (AN2), N-(4-hydroxy-3-methoxybenzyl) butanamide (AN3), N-(4-hydroxy-3-methoxybenzyl) hexanamide (AN4), and N-(4-hydroxy-3-methoxybenzyl) tetradecanamide (AN5), on cultured hepatic metabolizing cells of *Rattus norvegicus* (HTC) using the MTT assay.

2. Materials and Methods

2.1. Cell line

HTC cells, derived from *Rattus norvegicus* hepatoma, were obtained from the Cell Bank of Rio de Janeiro, Brazil. They were grown in 25-cm^2 culture flasks containing 10 mL of DMEM culture medium (Invitrogen - Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen - Carlsbad, CA, USA) and 1 mL/L of antibiotic/antimycotic solution and kept in a BOD oven at 37º C.

2.2. Treatment solution

Were tested the samples of capsaicin and analogs with lower pungency content (Table 1), considering the size of the acyl chain and the changes in the vanillyl ring proposed by Castillo et al. (2007), i.e., the number of carbons present in the chain (Radical R) and the radicals attached to the vanillyl ring (Radical X1 and X2). The radicals attached directly to the vanillyl ring (X1 and X2) varied between methoxy (MeO-), hydroxyl (-OH), and a combination of both (AN2, AN3, AN4, and AN5), or even just hydrogen (AN1). Regarding the size of the carbon chain, the analogs vary between 3 carbons (AN1, AN2, and AN3), 5 carbons (AN4), and 13 carbons (AN5).

The samples were diluted in dimethylsulfoxide (DMSO) (Alphatec, Brazil), not exceeding the final concentration of 0.1%, PBS (saline), and culture medium.

2.3. MTT assay

The MTT cytotoxicity assay [3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide] followed the protocol suggested by Mosmann (1983) with modifications. 96-well cell culture plates were used, where 2.0 x 10^4 HTC cells were seeded in each well, except for the control wells without cells (white).

The cells were cultured for 24 hours with 100 µL of culture medium. After this period, the culture medium of the plate was discarded and 100 µL of new medium were added to the groups negative control (CO-) (culture medium), positive control with the cytotoxic agent methyl methanesulfonate (MMS) (CO +) (concentration end of 150 µM), and treatments with concentrations of 10, 25, 50, 75, 100, 125, 150, 175 and 200 µM of each of the compounds (CAP, AN1, AN2, AN3, AN4, and AN5).

Table 1. Chemical composition of capsaicin and its analogs tested.

Compound	Modification in the vanillyl ring	Size of the acyl chain	Abbreviation
Capsaicin (8-methyl-N-vanylyl-6-nonenamide)	X_1= MeO -	R= C_9H_{17}	CAP
	X_2= HO -		
N-Benzylbutanamide	X_1= H -	R= C_3H_7	AN1
	X_2= H -		
N-(3-methoxybenzyl) butanamide	X_1= MeO -	R= C_3H_7	AN2
	X_2= H -		
N-(4-hydroxy-3-methoxybenzyl) butanamide	X_1= MeO -	R= C_3H_7	AN3
	X_2= HO -		
N-(4-hydroxy-3-methoxybenzyl) hexanamide	X_1= MeO -	R= C_5H_{11}	AN4
	X_2= HO -		
N-(4-hydroxy-3-methoxybenzyl) tetradecanamide	X_1= MeO -	R= $C_{13}H_{27}$	AN5
	X_2= HO -		

The cells were incubated for 24 and 48 hours and then the culture medium was replaced by 100 µL of serum-free medium plus MTT at a concentration of 0.167 mg mL^{-1}. The plate was incubated for another 4 hours and, in the sequence, the medium containing MTT was discarded. 100 µL of DMSO were added to the wells to dilute the formazan crystals formed.

The reading was performed in a microplate reader (FlexStation) at 550 nm.

2.4. Data analysis

The mean absorbances obtained in the three biological repetitions were compared by one-way analysis of variance (one-way ANOVA), followed by the Dunnet test ($\alpha = 0.05$, $p < 0.05$, $n = 3$) using the Action Stat Program. The percentage values of cell viability (VC) were estimated by Equation 1 (VC = Cell viability (%); ABS_T = Mean absorbance of treatment; ABS_C = Mean absorbance of the negative control).

$$VC = \left(\frac{ABS_T}{ABS_C} \right) \times 100 \quad (1)$$

3. Results and Discussion

The results in Figure 1-A show that over 24 hours the concentrations of 100, 150, 175, and 200 µM of capsaicin showed mean absorbances that were statistically lower and different from the negative control and thus had a cytotoxic effect on hepatoma cells of *Rattus norvegicus*. In fact, for the highest concentrations (> 125 µM), a dose-dependent cytotoxic effect can be observed, with cell viability reaching 74.99% (200 µM) (Table 2). Skrzypski et al. (2014) corroborate these results, as they demonstrated that capsaicin also decreased the viability and proliferation of pancreatic neuroendocrine tumor cells after 24 hours of exposure, depending on the dose, with concentrations varying between 10 and 200 µM.

These cytotoxic effects related to capsaicin, as demonstrated by other authors, may have occurred through the production of reactive oxygen species (ROS). This would stop the cell cycle, regulating the expression of the transcription factor and changes in the transduction signal pathways, cell growth, and survival, resulting in decreased viability and consequent cell death (Lewinska et al., 2015; Gómez-Sierra et al., 2013).

It is worth noting that the biological responses of capsaicin are directly influenced by its pharmacokinetic properties (Freitas et al., 2018). According to Saria et al. (1983), most capsaicin undergoes hepatic metabolism, and the increase in capsaicin metabolism by liver enzymes of the cytochrome P450 family (CYP450) results in a decrease in its half-life (Suresh and Srinivasan, 2010). This may be the justification for the fact that, within 48 hours

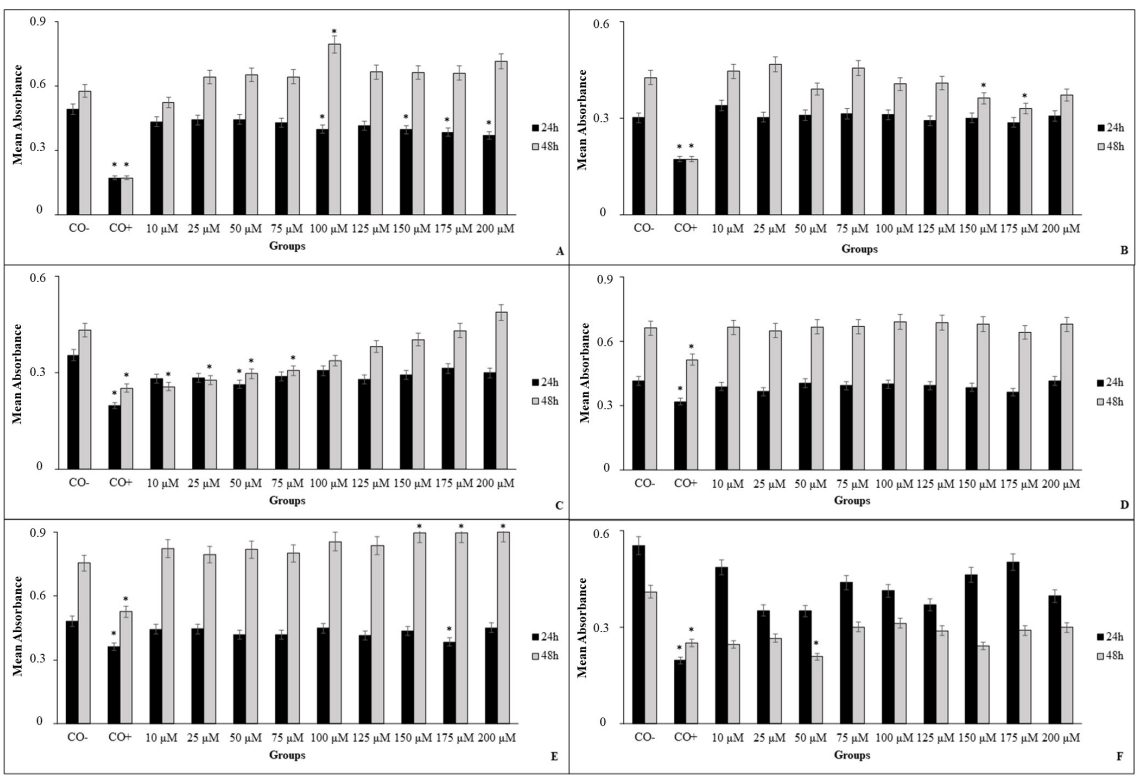

Figure 1. Mean absorbances of *Rattus norvegicus* hepatoma cells (HTC) from the negative (CO-) and positive (CO+) controls and treated with different concentrations of capsaicin (A), N-Benzylbutanamide (B), N-(3-methoxybenzyl) butanamide (C), N-(4-hydroxy-3-methoxybenzyl) butanamide (D), N-(4-hydroxy-3-methoxybenzyl) hexanamide (E) and N-(4-hydroxy-3- methoxybenzyl) tetradecanamide (F), for 24 and 48 hours. * Statistically significant result compared to the negative control ($p < 0.05$).

Table 2. Percentage of cell viability (VC) of *Rattus norvegicus* hepatoma cells (HTC), from controls and treated with different concentrations of Capsaicin (CAP), N-Benzylbutanamide (AN1), N-(3-methoxybenzyl) butanamide (AN2), N-(4-hydroxy-3-methoxybenzyl) butanamide (AN3), N-(4-hydroxy-3-methoxybenzyl) hexanamide (AN4) and N-(4-hydroxy-3-methoxybenzyl) tetradecanamide (AN5), for 24 and 48 hours.

Groups	VC [%]											
	CAP		AN1		AN2		AN3		AN4		AN5	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
CO-	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CO+	34.83	29.74	57.27	40.45	55.84	58.27	76.64	77.59	75.04	69.76	35.62	6131
10 µM	88.04	90.63	112.58	104.64	79.31	59.10	93.33	100.71	91.93	109.00	87.83	60.16
25 µM	89.51	111.41	100.52	109.63	80.34	64.18	87.78	98.25	92.31	105.43	63.62	64.84
50 µM	90.10	113.15	102.68	91.58	74.36	68.67	97.56	100.95	86.57	108.53	63.35	51.03
75 µM	86.84	111.43	104.38	106.87	81.43	70.79	94.63	101.19	86.96	106.22	79.31	73.30
100 µM	80.93	137.77	103.18	95.16	86.56	77.83	96.26	104.23	92.97	113.43	74.62	76.16
125 µM	84.05	115.30	96.98	95.95	78.75	88.26	94.60	104.05	86.28	111.03	66.86	70.50
150 µM	80.28	114.87	99.89	84.89	82.78	93.15	92.39	102.78	90.39	118.66	83.72	58.94
175 µM	77.94	114.36	95.28	77.55	88.50	99.73	87.21	97.12	79.50	118.87	90.71	70.62
200 µM	74.99	123.83	101.89	87.35	84.51	112.80	100.00	102.60	93.35	119.43	71.82	73.11

CO-: Negative Control; CO +: Positive Control.

(Figure 1-A), none of the tested capsaicin concentrations maintained the cytotoxic effect for HTC cells. In fact, the concentration of 100 µM had a stimulating effect on cell proliferation, with cell viability significantly greater than 100% (Table 2). According to Caetano et al. (2018), capsaicin increased the expression of the nuclear factor kappa B (NF-κB) and this was able to stimulate cell growth and induce the proliferation of colon carcinoma cells (Gómez-Sierra et al., 2013).

For AN1 (N-benzylbutanamide) (Figure 1-B), within 24 hours, no concentration had a cytotoxic effect on HTC cells. However, within 48 hours, concentrations of 150 and 175 µM showed lower mean absorbances than that of the negative control and, thus, cytotoxic effect. AN1 has as chemical modifications a reduction in the number of carbons in the acyl chain (from 9 of capsaicin to 3 in AN1) (Table 1) and the absence of the MeO- (methoxy radical) and -OH (hydroxyl radical) structures in the vanillyl ring. These modifications must have been responsible for the cytotoxic effect of these concentrations in a longer exposure time (48 hours) when compared to the effect presented by them in 24 hours.

AN2 (N- (3-methoxybenzyl) butanamide) also has only 3 carbons in the acyl chain (Table 1), as does AN1, but the vanillyl ring of AN2 lacks the hydroxyl radical (-OH) but has the methoxy radical (MeO-) as in capsaicin. These modifications were possibly the most efficient for the cytotoxic effect for the HTC cells of the present study since, within 24 hours, AN2 (Figure 1-C) presented a cytotoxic effect for the metabolizing cells at a concentration of 50 µM, with cell viability of 74.36% (Table 2). Within 48 hours, the lowest concentrations (10, 25, 50, and 75 µM) of AN2 had a toxic effect on HTC cells, with cell viability below 71%. Thus, the absence of the hydroxyl radical in the vanillyl ring, which is a highly damaging oxidizing radical for most biomolecules (Li et al., 2020), may be involved with cytotoxicity in the longer exposure time of HTC cells, whereas the presence of the methoxy radical (MeO -) allowed AN2 to be cytotoxic in the time of 24 and 48 hours from the lowest concentrations.

On the other hand, only reducing the acyl chain to 3 carbons, without modifying the vanillyl ring, as in the case of AN3 (N-(4-hydroxy-3-methoxybenzyl) butanamide) (Table 1), besides not having the same effect of capsaicin and the analogs AN1 and AN2, resulted in the absence of cytotoxic effects of this compound for HTC cells in the two evaluated times (24 and 48 hours) (Figure 1-D), with cell viability above 87% (24 hours) and 97% (48 hours) (Table 2). Thus, the reduction in the number of carbons in the acyl chain of AN3 decreased the size of the molecule and, probably, its pungency, as indicated by Castillo et al. (2007), but, on the other hand, prevented it from acting on any component or cell pathway that could cause cytotoxicity to the cells of the present study.

These results can be justified, as highlighted by Goel et al. (2008), by the fact that capsaicin and other bioactive components present their biological activities through direct physical interaction with one or more cellular proteins, such as cytokines, growth factors, transcription factors, and enzymes. Thus, changing the chemistry of its structures can influence/alter biological activity. Therefore, the indication that the cytotoxicity of these components is associated with an intermediate chain size and the presence of a radical such as methoxy, responsible for increasing the probabilities of interaction with other molecules, opens space for further research on variations of these analogs.

These data corroborate the result obtained with AN4 (N-(4-hydroxy-3-methoxybenzyl) hexanamide) (Figure 1-E). This analog has the same radicals as capsaicin

in the vanillyl ring and only a reduction in carbons in the acyl chain (from 9 in capsaicin to 5 in AN4) and was the one that showed the biological activity most similar to capsaicin. In this case, in 24 hours, the concentration of 175 µM was cytotoxic to HTC cells, with cell viability of 79.50% (Table 2) and, in 48 hours, there was a stimulus for cell proliferation for concentrations 150, 175, and 200 µM, with cell viability at least 18% greater than that of the negative control. Thus, it is evident that the chemical structure is indeed a crucial factor in the activity developed.

Furthermore, the increase in the carbon chain in the acyl group of the capsaicin molecule, such as AN5 (N-(4-hydroxy-3-methoxybenzyl) tetradecanamide) (Table 1), also resulted in different biological results. In this case, the concentration of 50 µM, within 48 hours (Figure 1-F) was statistically different from the negative control and, thus, had a cytotoxic effect. However, some concentrations of AN5 in 24 hours and all concentrations in 48 hours showed cell viability below 79% (Table 2), with a concentration of 50 µM reaching viability of 51.03%. The AN5 has 13 carbons in the acyl chain, a large number that is responsible for creating a region with strong nonpolar forces, contrasting with the strong polarity presented on the opposite side (vanillyl chain).

The results obtained in the present study are important because they prove that the chemical modifications of capsaicin are relevant since they change their usefulness and functions.

4. Conclusions

Our results show that changing the chemical structure of capsaicin influenced its cytotoxic activity. AN4 (N-(4-hydroxy-3-methoxybenzyl) hexanamide), the chemical compound most similar to capsaicin (reduction of 4 carbons) in the acyl group of the molecule, was the one with the most similar biological activity. Additionally, the removal of the hydroxyl radical from the vanillyl group of capsaicin, together with the reduction of the acyl carbon chain to 3 carbons, as in the case of AN2 (N-(3-methoxybenzyl) butanamide), resulted in the best biological activity, with cytotoxic effects for metabolizing cells in the lowest concentrations and in the two evaluated times. Increasing the carbon chain in the acyl group of the capsaicin molecule, which is the case of AN5, also had an evident effect in reducing the viability of HTC cells. Thus, our results prove that the chemical changes of capsaicin changed its biological activity.

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