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Colorectal cancer chemoprevention by S-allyl cysteine-caffeic acid hybrids: Pro-apoptotic effect, anti-inflammatory, *in vitro*/*in silico* antioxidant, Docking and Drug-Likeness studies.

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Abstract

Conventional chemotherapy for colorectal cancer (CRC) gives only a small increase in patient survival, since it is often diagnosed in late stages, when tumor has disseminated to other organs. Besides, it is common to observe that malignant cells acquire tumor escape mechanisms which leads to therapy resistance. Considering these facts, the discovery of new molecules with therapeutic potential has become an invaluable tool in chemoprevention. In this context, we previously evaluated two hybrids (**SAC-CAFA-MET** and **SAC-CAFA-PENT**) which exhibited selective cytotoxicity against SW480, with better results than the conventional chemotherapeutic agent (5-fluorouracil; 5-FU). Here, we investigated a little deeper in the possible mechanism of these molecules to identify potential therapeutic alternatives for the treatment of CRC. Both compounds induced cell damage and reduced ROS formation. Further evaluations showed that **SAC-CAFA-MET** induces cell death independent from caspases and p53, but probably mediated by the negative regulation of the proapoptotic Bcl-2. In addition, the lack of activation of caspase 8 and the positive regulation of caspase 3 induced by **SAC-CAFA-PENT** suggest this compound acts through an apoptotic mechanism, probably initiated by intrinsic pathway. Besides, the down regulation of IL-6 by **SAC-CAFA-PENT** suggests it also induces a significant anti-inflammatory process. In addition, docking studies would suggest caspase-3 modulation as the primary mechanism by which hybrids elicits apoptosis in human colorectal adenocarcinoma SW480. Meanwhile, DFT calculations suggest that hybrids would produce effects in modulation of ROS in SW480 cells via hydrogen atom transfer pathway (HAT). Finally, both, **SAC-CAFA-MET** and **SAC-CAFA-PENT** displayed a favorable pharmacokinetic profile. The current work highlights the potential of the lead compounds **SAC-CAFA-MET** and **SAC-CAFA-PENT** as potential agents for colorectal cancer chemoprevention.

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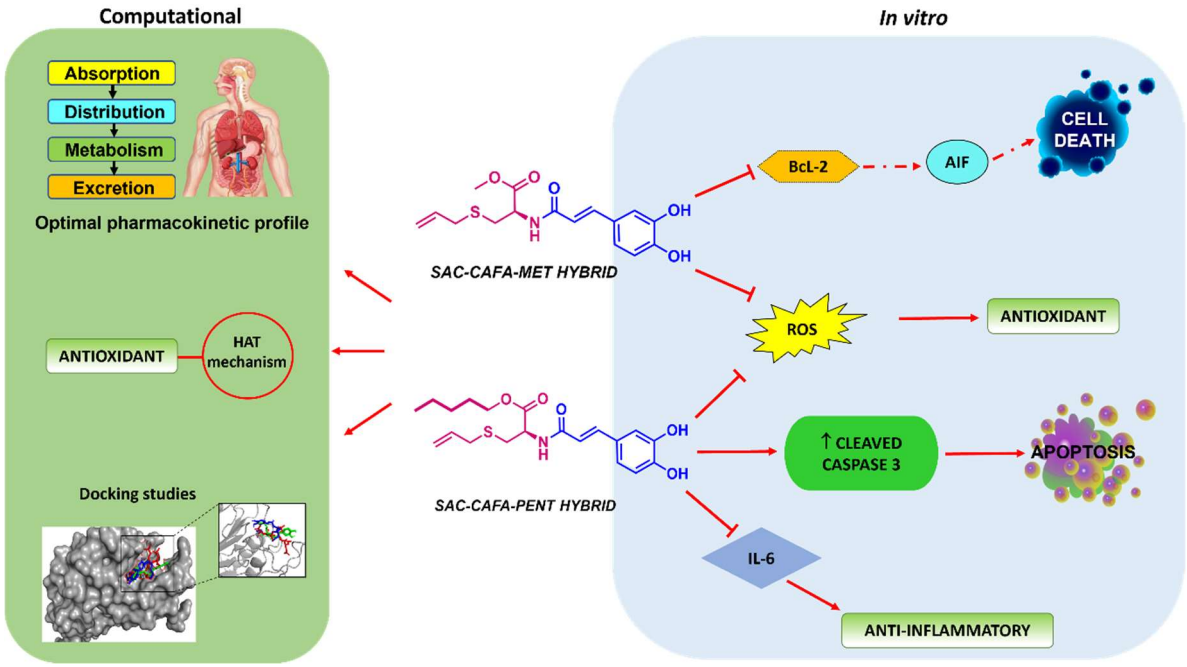
Graphical Abstract



Keywords: *S*-allylcysteine, caffeic acid, hybrid compounds, chemoprevention, colorectal cancer, cell death, apoptosis, anti-inflammation, antioxidant, *in silico*, docking.

Abbreviations: SAC: *S*-allylcysteine; CA: Caffeic Acid; CRC: Colorectal Cancer; DMSO: Dimethyl sulfoxide; 5-FU: 5 Fluorouracil; ROS: Reactive Oxygen Species.

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Introduction

Colorectal cancer (CRC) is considered one of the main cancers with modifiable causes, that can be highly preventable by living a healthy lifestyle, through minimizing exposure to risk factors like avoiding the use of tobacco and alcohol, maintaining a healthy body weight, practicing physical activities, including a diet low in red and processed meats and high in fiber, vegetables, and fruits [1,2]. Despite this, it is still a leading cancer-related cause of death worldwide, being the second most common counting about 935.173 deaths in 2020, only preceded by lung cancer, with an estimated of 1'931.590 new cases in the same year [3,4]. Due to the widespread occurrence of the risk factors and the increase in the statistics, extensive research is ongoing to develop new agents as potential therapeutic alternatives against colorectal cancer.

Current treatments for CRC include different regimens with chemotherapeutic agents alone or in combination, for example, FOLFOXIRI (folinic acid/5-FU/oxaliplatin/irinotecan) FOLFIRI (folic acid/5-FU/irinotecan), FOLFOX (5-FU/leucovorin/oxaliplatin), which are composed of 5-fluorouracil as the backbone of the treatment, and XELIRI/CAPIRI (Capecitabine with irinotecan), XELOX/CAPEOX (Capecitabine with oxaliplatin), which are composed of an oral form of 5-fluorouracil, capecitabine. Although these conventional treatments are effective, all they cause high-grade toxicity including neurological disorders, gastrointestinal side-effects, myelosuppression, neutropenia, anemia, among others, which many times result in dose limitations or cessation of the anticancer therapy [5-7]. Considering this, the search for new therapeutic alternatives becomes necessary. Thereon, chemoprevention has been explored for management of different cancers, increasing the interest in understanding the biology of carcinogenesis to identify molecular targets to disturb this process. This strategy is based on the use of natural, synthetic, or biological agents to reverse, suppress or either prevent the steps in tumor initiation, promotion, or progression [8,9]. The design and discovery of novel drug candidates represents the initial and hence probably the most crucial step in the drug development process since the identification of hits and subsequent lead structures is a very risky and expensive process. On the other hand, it is well recognized that many diseases are caused for defects in different biological targets,

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involving a plethora of biochemical and physiological processes that often even occur simultaneously. Besides, it is common that even the most promising hits will only influence one biological target and it would probably not be sufficient to effectively combat multifactorial diseases like cancer. Because of this, the use of agents with different mechanisms of action is one of the methods adopted for treating this disease [10,11]. Thereon, the use of hybrid compounds has emerged as promising strategy in medicinal chemistry and drug discovery research since these compounds combine two distinct biologically active molecules into one entity that could act in different targets, displaying dual effect [12-16]. We have previously reported the biological activity of *S*-allyl cysteine ester–caffeic acid amide hybrids using a colon cancer cell line. According to those results, some hybrids induced mitochondrial depolarization and cell cycle arrest in G2/M or S-phase, suggesting that these molecules could exert either a cytotoxic or cytostatic effect in SW480 cell line [17]. In this study we studied the most active molecules to provide a better approach to the possible mechanism of action of these hybrid molecules to identify possible therapeutic alternatives for the treatment of colorectal cancer.

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Results and discussion

Considering that conventional chemotherapy induces several side effects which many times result in dose limitations or cessation of the anticancer therapy [5,6], the search for new therapeutic alternatives becomes necessary. The aim of the present study was to evaluate the effect of two *S*-allyl cysteine ester - caffeic acid amide hybrids in the immunomodulation and cell stability of human adenocarcinoma colon cells respect to changes on ROS production, apoptotic proteins, inflammation related biomarkers, as well as the effect in the activity of matrix metalloproteinase 7 (*MMP*-7) and *MMP*-9. In a previous study we found that, the hydroxylated compounds SAC-CAFA-MET and SAC-CAFA-PENT (in the precedent publication named **9a** and **9e**, respectively) displayed great selective activity with better results than starting compounds and the reference drug 5-FU [17]. Here we make an approach to the possible mechanisms involved in the activity of those hybrids to induce cell death using human colon cancer cells (SW480).

Source of hybrid molecules

The synthesis of the hydroxylated compounds SAC-CAFA together with the full characterization and other biological activities, were previoulsy reported by Castrillon et al (2019) [17]. Scheme 1 shows a brief description of the route used for the synthesis of the molecules. The reaction of nucleophilic sustitution between cysteine (**1**) and allyl bromide gived the S-Allyl cysteine (**2**) which was esterified with the corresponding alcohol in the presence of thionyl chloride, obtaining compound **3**. On the other hand, the phenolic hydroxyl groups of caffeic acid were protected as TBDMS providing compound **5**. Then, compounds **3** and **5** were submitted to peptide type-coupling using HBTU as amide bond promoter giving compound **6**. Finally, hybrids **SAC-CAFA** were obtained by deprotection from compound **7**.

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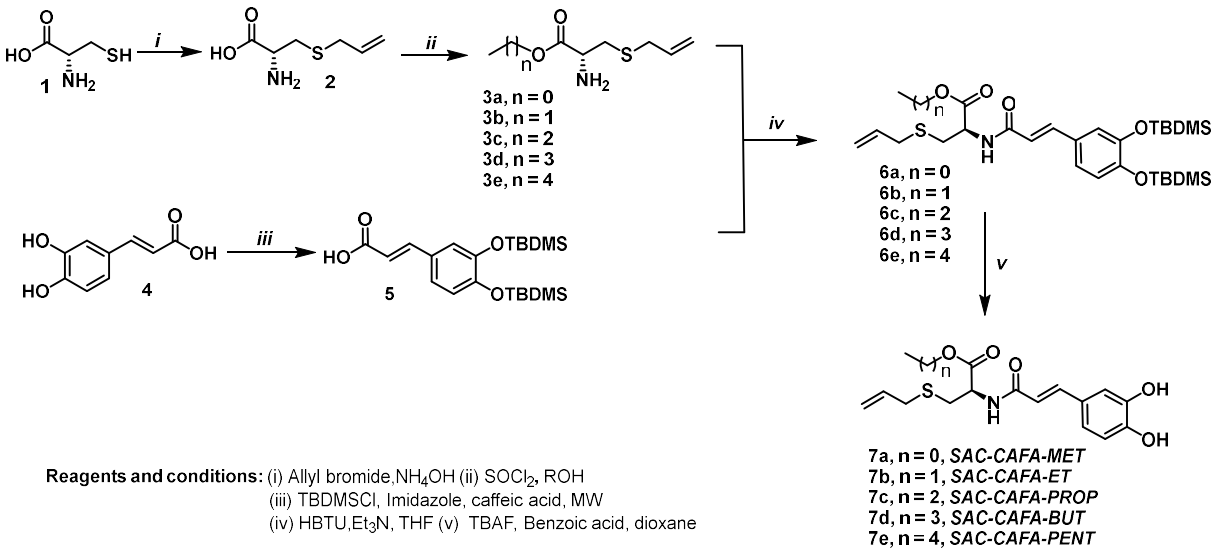

Scheme 1. Synthesis of hybrids SAC-CAFA. This data were taken from the previous report of the process made by Castrillon et al (2019).

Effect of SAC-CAFA-MET and SAC-CAFA-PENT on Reactive Oxygen Species (ROS)

There is evidence that supports the role of oxidative stress and ROS (hydrogen peroxide, hydroxyl radical, superoxide anion and peroxynitrite) in genotoxicity and the development of colon cancer. When ROS are produced in low doses, they play a role in physiological functions, and they are essential for regulation of various cellular signaling pathways. However, high levels of ROS may react with biomolecules (lipids, carbohydrates, proteins, and nucleic acids) interfering with cell function. This resulting oxidative damage is the first step involved in mutagenesis and carcinogenesis [18,19]. On the contrary, increasing antioxidant activity is seen as a potential strategy to delay the harmful effects of ROS [18,20]. Because of this, we investigated if SAC-CAFA-MET and SAC-CAFA-PENT could have an effect in modulation of ROS in SW480 cells. The results showed that both hybrids significantly reduced ROS formation regarding the control, suggesting they could act as antioxidants in this model (Figure 1). This is consistent with previous information reported for the parental compounds alone. Thus, different authors have reported that *S*-allylcysteine (SAC) possesses strong antioxidant activity by scavenging intracellular ROS [21-23]. Similarly, it was reported that SAC reduced oxidative stress in a rat model of focal cerebral ischemia [24], a sporadic Alzheimer's disease model [25] and a mouse model of Parkinson's

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disease [26]. Alternatively, different authors have also reported that caffeic acid (CA) possesses potential anticancer activity with well-defined pharmacological mechanisms associated with the inhibition of ROS production [8]. This information supports our findings, suggesting that one possible mechanism of the hybrid molecules SAC-CAFA-MET and SAC-CAFA-PENT could be potentiated by the inhibition of oxidative stress in SW80 cells.

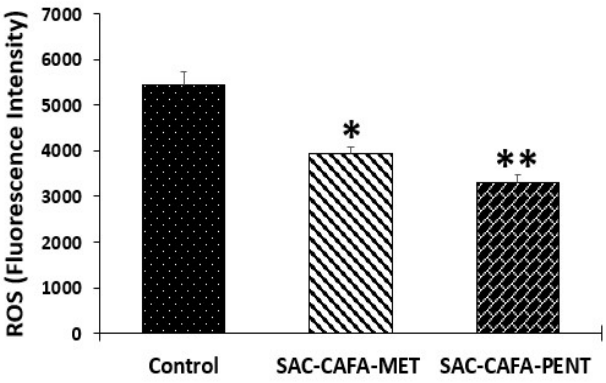

Figure 1. Intracellular ROS induced by hybrids SAC-CAFA-MET (124.2 µM) and SAC-CAFA-PENT (118 µM) or DMSO 1% (control) in SW480 cells. Fluorescent dye CM-H2DCFDA was used. Data are presented as the mean ± SE of three independent experiments. *p*-values lower than 0.05 were considered statistically significant (*$p<0.05$; **$p < 0.01$).

Effect of SAC-CAFA-MET and SAC-CAFA-PENT in the expression of caspases-3, -7 and -8.

In normal conditions, the apoptotic pathway is a highly regulated process that culminates in the death of a cell, involving different anti-apoptotic and pro-apoptotic proteins together with the sequential activation of proteases called caspases, responsible for initiating the hallmarks of the degradation phase of apoptosis including cell shrinkage, membrane blebbing and DNA fragmentation [27]. Abnormalities in apoptotic function contribute to both the pathogenesis of colorectal cancer and its resistance to chemotherapeutic drugs. Thus, the design of different anticancer drugs has been focused on the modulation of the different molecules to develop more selective agents with increased efficacy and reduced side effects. To investigate if the mechanism of action of SAC-CAFA-MET and SAC-CAFA-PENT is related to apoptosis in SW480 cells, different biomarkers were evaluated.

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Caspases can be sub-classified as initiator (caspase-8 and -9) or executioner (caspase-3, -6, and -7) [28]. All these proteases are synthesized as proenzymes and require a highly regulated process to be activated. Once initiator caspases undergo self-activation, they can activate the executioner caspases to start the hydrolysis of different proteins from the cytoskeleton, nuclear proteins, and other molecules to initiate the final process of death [29]. Because of this, we tested the effect of SAC-CAFA-MET and SAC-CAFA-PENT in the levels of some relevant caspases, such as caspase-3 (Figure 2A), caspase-7 (Figure 2B) and caspase-8 (Figure 2C), to determine the effect on SW480 cells after 48 hours of treatment. First, it was evaluated the executioner caspase-3, which is considered one of the most important proteins, acting as the primary executioner of apoptotic death doing this process more efficient [27,30]. According to the results, we found that SAC-CAFA-PENT induced a significant increase in the active form of this protease, suggesting a possible mechanism related to apoptosis in this *in vitro* model. Besides, considering the previous results reported by our group according to the changes induced by this compound in mitochondrial membrane permeability [17] and the absence in the activation of caspase-8, we hypothesized that apoptosis induced by this hybrid is not triggered by extrinsic pathway. On the other hand, the lack of activation of caspase-3 by SAC-CAFA-MET suggests that this molecule could involve another type of programmed cell death (PCD) which is supported by the previous evidence that reports that PCD can occur in complete absence of caspase activation [31,32]. In addition, we observed that none of the compounds caused changes on the levels of caspase -7 and -8, which supports the idea that SAC-CAFA-MET involves a different mechanism, not related to apoptosis.

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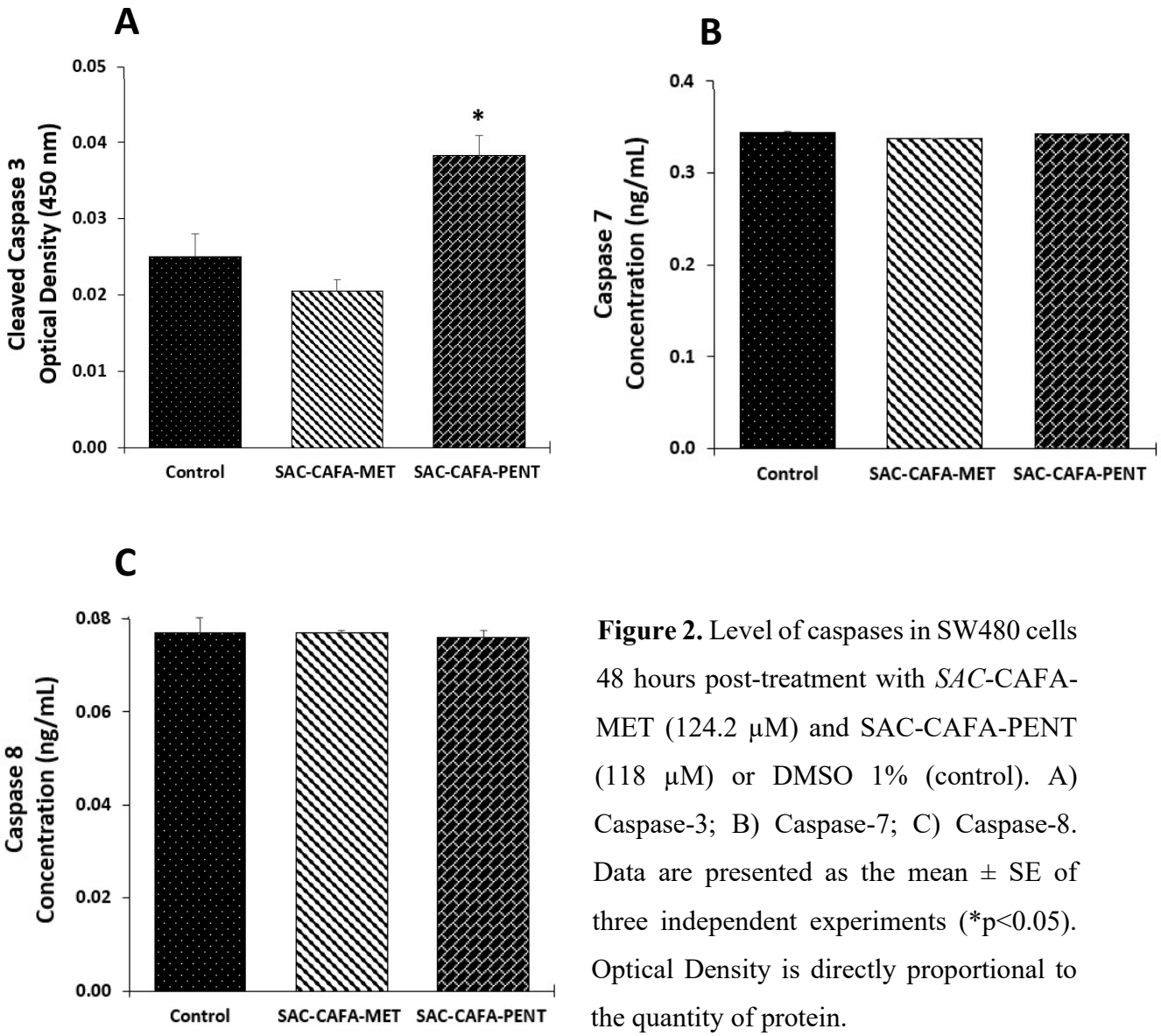

Figure 2. Level of caspases in SW480 cells 48 hours post-treatment with *SAC*-CAFA-MET (124.2 µM) and SAC-CAFA-PENT (118 µM) or DMSO 1% (control). A) Caspase-3; B) Caspase-7; C) Caspase-8. Data are presented as the mean ± SE of three independent experiments (*$p<0.05$). Optical Density is directly proportional to the quantity of protein.

Effect of SAC-CAFA-MET and SAC-CAFA-PENT in the expression of apoptotic biomarkers

Several apoptotic biomarkers play pivotal roles in different cellular processes. The BcL-2 family is involved in the regulation of apoptosis and therefore plays a vital role in protecting against cancer. Among the genes involved in apoptotic pathway, anti-apoptotic Bcl-2 contributes to cancer development and progression by promoting the survival of malignant cells. Thus, it is a prime target for novel specific anti-cancer therapies [33,34]. The effect of

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SAC-CAFA-MET and SAC-CAFA-PENT was tested in SW480 cells 48 hours after treatment and results revealed that SAC-CAFA-MET causes a significant down regulation of BcL-2 (Figure 3A), besides, considering that this protein suppresses the release of Apoptosis Inducing Factor (AIF) from mitochondria, authors hypothesized that the negative modulation of this anti-apoptotic biomarker could probably be implicated in a caspase-independent death process mediated by AIF, causing the loss of mitochondrial membrane potential and the canonical changes of cell death characterized by chromatin condensation, and DNA fragmentation [35-38]. This is also consistent with previous studies reported by our team, where this hybrid displayed mitochondrial depolarization [17]. Other authors carried out a different study using one of the precursors of the hybrids evaluated (*S*-allylcysteine; SAC) and they reported that it alone suppressed the proliferation of prostate cancer cells through down regulation of bcl-2 [39]. On the other hand, it was observed that human bid levels were not different from the control (Figure 3B). This was related to other results since bid is cleaved by caspase-8 after a death signal mediated by death receptors and none of the hybrids caused changes in caspase 8 expression [40]. In addition, considering that p53 has an essential role in regulating cell death [41,42], the effect of SAC-CAFA-MET and SAC-CAFA-PENT was tested (Figure 3C). According to the results, authors found that none of the hybrids evaluated caused significant changes in this protein, which complements the information that these hybrids caused tumor cell death through molecular mechanisms independent of p53. Other authors reported similar results with different caffeic acid esters, suggesting that an active component extracted from honeybee propolis, named caffeic acid phenethyl ester (CAPE) induces apoptosis through both p53-dependent and p53-independent pathways [43].

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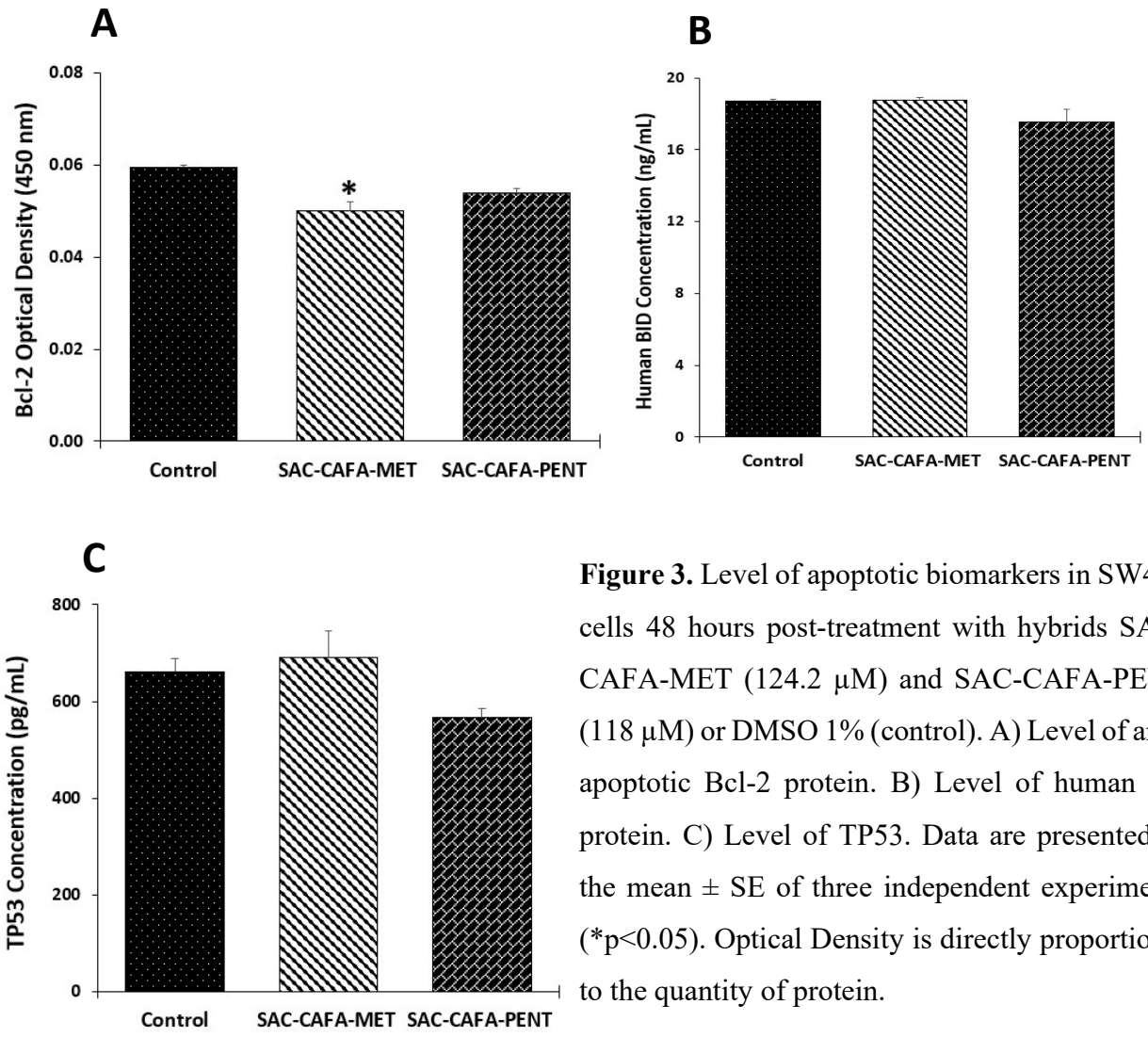

Figure 3. Level of apoptotic biomarkers in SW480 cells 48 hours post-treatment with hybrids SAC-CAFA-MET (124.2 µM) and SAC-CAFA-PENT (118 µM) or DMSO 1% (control). A) Level of anti-apoptotic Bcl-2 protein. B) Level of human bid protein. C) Level of TP53. Data are presented as the mean ± SE of three independent experiments (*$p<0.05$). Optical Density is directly proportional to the quantity of protein.

Effect of hybrids SAC-CAFA-MET and SAC-CAFA-PENT in the activity of matrix metalloproteinase 7 (MMP-7) and MMP-9

The extracellular matrix (ECM) is composed of different macromolecules and minerals, providing a structural and biochemical support for the cells, regulating both inter- and intra-cellular signaling for different processes as differentiation, adhesion, and invasion. In cancer, malignant cells interact with the ECM causing structural remodeling, to facilitate migration from a primary tumor site. Different proteins are involved in the ECM remodeling and degradation. Thereon, matrix metalloproteinases (MMPs) have been extensively investigated for acting as

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proteolytic enzymes, with ability to degrade all components of the ECM. Among them, MMP7 and MMP9 have long been evaluated in colorectal cancer given the existence of a correlation between an increase in these endopeptidases and CRC invasion [44,45]. Because of that, authors decided to evaluate if hybrids SAC-CAFA-MET and SAC-CAFA-PENT could regulate the activity of these enzymes in SW480 cells. According to the results, none of the compounds caused changes in modulation of MMP7/9 (Figure 4A/B), suggesting a different mechanism of action.

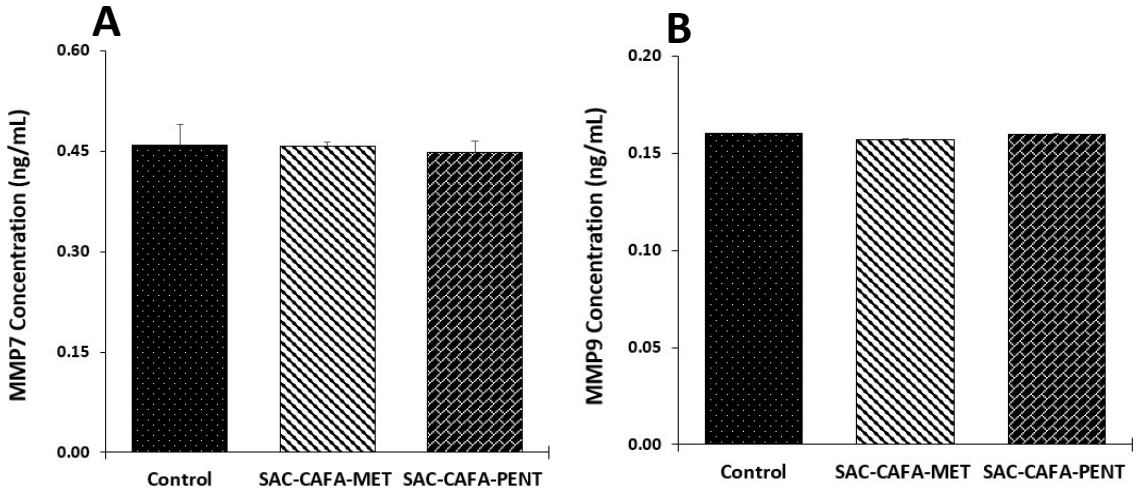

Figure 4. Concentration of matrix metalloproteinase 7 (MMP7) and MMP9. Data are presented as the mean ± SE of three independent experiments.

Effect of hybrids SAC-CAFA-MET and SAC-CAFA-PENT on expression of interleuquin-6 (IL-6)

It has been observed that inflammation can contribute to CRC development and progression [46]. Thus, several studies have focusing on the evaluation of a variety of cytokines to observe if it is possible to modulate their expression. Some authors have reported an increase in the levels of interleuquin-6 (IL-6) in patients with colorectal cancer, which is related to the size of the tumor, the severity of the pathology and the survival rate [47]. Besides, they mentioned that down regulation

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in the expression of this cytokine prevents multiplicity and tumor progresion in intestinal cells [48,49]. For those reasons, the study of pro-inflammatory cytokines has become an important target in the discovering of molecules with potential activity against CRC. Considering these facts, we evaluated if hybrids SAC-CAFA-MET and SAC-CAFA-PENT could modulate the expression of IL-6 (Figure 5), and results displayed that only the second hybrid mentioned is able to induce a significant negative regulation of this biomarker, suggesting that it can also acts through anti-inflammatory mechanisms in this model of colorectal cancer. On the other hand, due to the important role of these cytokines in carcinogenesis, we also evaluated the response to cyclooxygenase 1 and 2(COX), prostaglandin E2, tumor necrosis factor α (TNF α) and interleukin 1β (IL-1 β). However, we observed that none of the hybrids evaluated can modulate the expression of these biomarkers (data not shown). Other authors have shown similar results when evaluated the precursors used for the synthesis of hybrids SAC-CAFA-MET and SAC-CAFA-PENT. For example, You et al (2013) [50] reported that SAC showed no inhibitory effect on COX-2 production using RAW264.7 monocyte/macrophage-like cells. Unlike this study, Kim et al (2013) [23] evaluated SAC and CA in a skin model, and they concluded that both compounds display anti-inflammatory activity through modulation of COX-2. Besides, Zarezadeh et al. (2017) [51] reported that SAC caused down regulation of IL-1β using male Wistar albino rats. All these findings show how different is the response between models and complement the possible mechanism of action associated to the hybrid molecules evaluated in this study.

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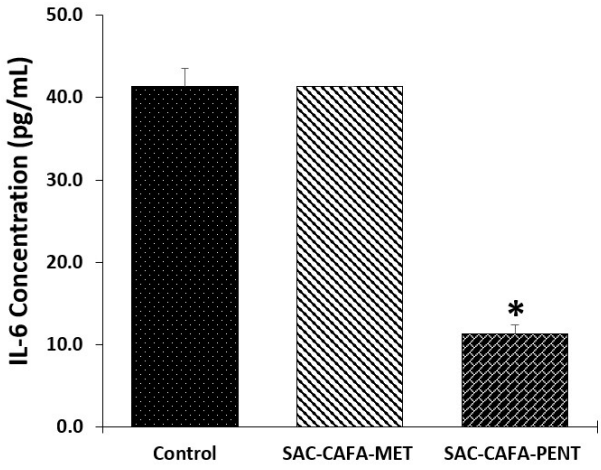

Figure 5. Level of pro-inflammatory cytokine IL-6 in SW480 cells 48 hours post-treatment with hybrids SAC-CAFA-MET (124.2 µM) and SAC-CAFA-PENT (118 µM) or DMSO 1% (control). Data are presented as the mean ± SE of three independent experiments (*p<0.05).

Multi-target docking studies and prediction of binding pose

Aiming to investigate the binding mechanism at a molecular level so as to present a reasonable explanation by which hybrids **6** (n= 0-4) including promising SAC-CAFA-MET and SAC-CAFA-PENT induced SW480 cell death, docking-based target-fishing strategy was performed. From the experimental findings, targeting proapoptotic caspase 3 and Bcl-2 proteins, as well the pro-inflammatory cytokine IL-6, may be responsible for the caused in vitro cytotoxic effect of SAC-CAFA-MET and SAC-CAFA-PENT in human colorectal adenocarcinoma SW480 cells. In this light, SAC-CAFA-MET and SAC-CAFA-PENT hybrids were docked into each catalytic domain of human X-ray crystallographic structures of caspase 3, Bcl-2 and IL-6 proteins via grid-based ligand docking with AutoDock Vina, and affinity scores along the best binding pose were estimated (Table 1). Interestingly, among these targeted proteins, hybrids **6** (n= 0-4) displayed greater binding affinity for human caspase-3 (PDB ID: 5I9B) than Bcl-2 (PDB: 4MAN) and IL-6 (PDB: 1ALU). In particular, the results indicated that hit-compounds SAC-CAFA-MET and SAC-CAFA-PENT binds to the caspase-3 with good binding energy close to −7.3 kcal.mol^{-1}, thereby suggesting these compounds could act into the catalytic domain of the enzyme to alter significantly its function. Therefore, we hypothesized that modulation of the activity of caspase-3 may be the primary biochemical mechanism by which SAC-CAFA-MET and SAC-CAFA-PENT inhibit SW480 cell growth.

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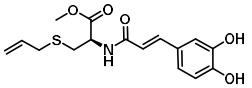
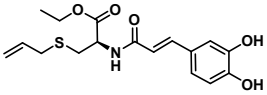
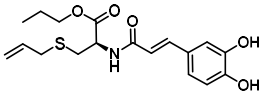
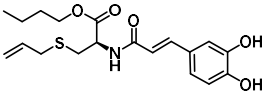
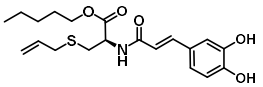
Table 1. Best binding energy (kcal.mol^{-1}) based on AutoDock scoring of the hybrids **6** taken from the previous report by Castrillon et al 2019 [17].

Entry	Structure	IC_{50} (mM)	IS	Target proteins (docking score, kcal.mol^{-1})		
				Caspase-3 (PDB ID: 5I9B)	Bcl-2[b] (PDB: 4MAN)	IL-6[c] (PDB: 1ALU)
6, n=0 (SAC-CAFA-MET)		0.12*	1.5	-7.2	-5.1	-5.3
6, n=1		0.12*	>83.33	-7.0	-5.1	-5.1
6, n=2		0.11*	>90.91	-7.1	-5.3	-5.0
6, n=3		0.15*	>66.67	-7.1	-5.2	-5.2
6, n=4 (SAC-CAFA-PENT)		0.12*	>83.33	-7.3	-5.4	-5.0
Venetoclax[a]		—	—	—	-10.9	—

[a]Potent and selective Bcl-2 inhibitor (EC_{50} = 4 nM; [b]Bcl-2: B-cell lymphoma-2; [c]Interleukin-6 receptor.

Caspases are a conserved family of cysteine-dependent proteases which are classically associated in the execution of apoptosis [52]. Among them, caspase-3 plays an important role in regulating and implementing the cell death program during apoptosis event. Caspase-3 substrate-binding region is characterized by the presence of amino acids residues, Arg64, Leu119, Ser120, His121, Gln161, Ala162, Cys163, Ser198, Tyr204, Ser205, Trp206, Asn208, Ser209, Trp214, Ser249, Phe250, Ser251 and Phe252 [53]. Thus, we explored the binding interactions of the promising SAC-CAFA-MET (in green) and SAC-CAFA-PENT (in blue) into the Caspase-3 receptor (PDB: 5I9B) in that domain. The docking results showed that in addition to the hybrids bind to Caspase-3 with a good binding affinity in about −7.3 kcal.mol^{-1}, they fitting-well in the active pocket of Caspase-3, as illustrated in Figure 6. Indeed, the best docking conformations obtained for these hybrids were in good agreement to that X-ray crystallographic pose of the peptide-based inhibitor Ac-DEVD-CMK (in red).

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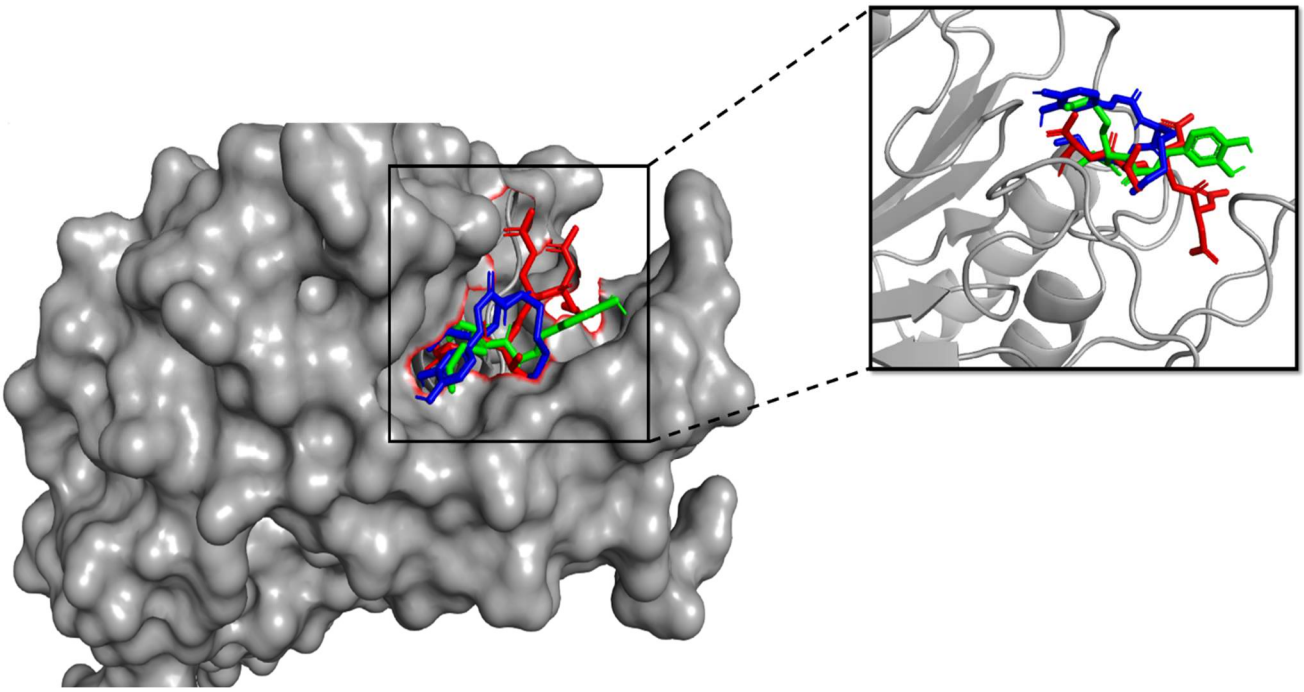

Figure 6. Superposition of the best docked conformations of hybrids SAC-CAFA-MET (green) and SAC-CAFA-PENT (blue), and crystallographic binding mode of inhibitor Ac-DEVD-CMK (red) within the Caspase-3 binding domain.

This particular result supports our proposal: SAC-CAFA-MET and SAC-CAFA-PENT might block caspase-3 function with good binding affinity preventing the cell growth and proliferation. This preliminary finding was also supported by a visual inspection of the 2D protein/ligand interaction plot after the docking run (Fig. 7A,B), which revealed that SAC-CAFA-MET and SAC-CAFA-PENT would form key hydrogen bond interactions with His121, Glu123, Arg207, Thr62 and Gly122 residues which play an important role in the Caspase-3 function. Besides, one π-anion interaction is seen between the aromatic moiety on SAC-CAFA-MET and the essential charged residue Glu123 in the target protein. Furthermore, both hybrids show interactions by π-contacts with the residuals around the catalytic domain of the enzyme, such as Tyr204, Trp206 and Phe256. Finally, multiples hydrophobic interactions surrounded by side chains into the binding cleft were observed between hybrids and caspase-3, which potentially stabilize protein-ligand complex upon the binding event. These computational findings suggest that a probable modulation of Caspase-

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3 would be the primary action mechanism for explaining the in vitro cytotoxic response registered for SAC-CAFA-MET and SAC-CAFA-PENT hybrids in SW480 colon cancer cells, which are in good agreement with the experimental data.

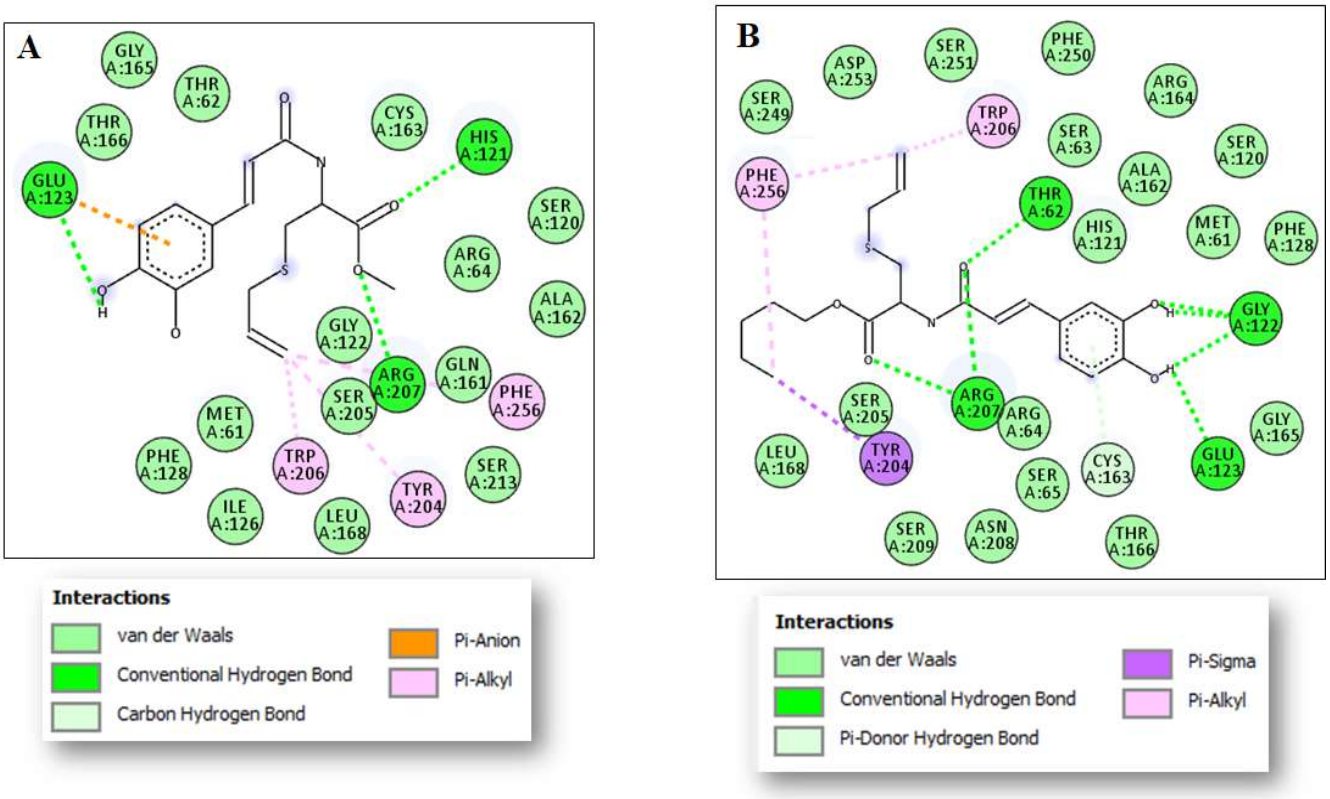

Figure 7. A. 2D protein-ligand interaction plot after docking procedure for SAC-CAFA-MET. **B.** 2D ligand-receptor interaction diagram for SAC-CAFA-PENT. Dashed lines represent H-bonds and π-contacts.

DFT analysis on the radical scavenging activity of SAC-CAFA-MET and SAC-CAFA-PENT

Excess formation of reactive free radicals by various enzymatic and non-enzymatic processes in the body has been found associated the initiation and progression of various cancer types. In neoplastic disorders, reactive oxygen species (ROS) overproduction contributed in different stage of carcinogenesis event such as cancer development and progression. Therefore, investigations addressed on new compounds expressing antioxidant and free radical-scavenging effects play a key role in cancer drug development. In this paper, we founded that SAC-CAFA-MET and SAC-CAFA-PENT could have an effect in modulation

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of ROS in SW480 cells in vitro. These findings might suggest that, apart from caspase-3 modulation, a second possible mechanism for the cytotoxic response of hybrids via interrupting cell antioxidant detrimental effects could be also implicated. Thus, in order to further understand above mentioned experimental antioxidant properties for hit hybrids, quantum-chemical calculations based on the density functional theory (DFT) approach was employed to investigate the energetics behavior of their free radical scavenging reactions. First at all, three of the most favorable antioxidant mechanisms were investigated as follow: hydrogen atom transfer (HAT), single electron transfer-proton transfer (SET-PT), and sequential electron transfer – proton transfer (SET-PT) in aqueous medium and in the gas phase.

i) Hydrogen atom transfer (HAT):

$$R_1OH + R_2^{\bullet} \rightarrow R_1O^{\bullet} + R_2H \quad (1)$$

ii) Sequential proton transfer – electron transfer (SPT-ET):

$$\textbf{Step 1}\ R_1OH + R_2^{\bullet} \rightarrow R_1O^{-} + R_2H^{\bullet+} \quad (2)$$

$$\textbf{Step 2}\ R_1O^{-} + R_2H^{\bullet+} \rightarrow R_1O^{\bullet} + R_2H \quad (3)$$

iii) Sequential electron transfer – proton transfer (SET-PT):

$$\textbf{Step 1}\ R_1OH + R_2^{\bullet} \rightarrow R_1OH^{\bullet+} + R_2^{-} \quad (4)$$

$$\textbf{Step 2}\ R_1OH^{\bullet+} + R_2^{-} \rightarrow R_1O^{\bullet} + R_2H \quad (5)$$

As shown in Table 2, DFT analysis of SAC-CAFA-MET and SAC-CAFA-PENT suggest that these compounds would react with free radicals via hydrogen atom transfer (HAT), which in turns may be used to explain antioxidant response for hybrids in vitro. Thus, when the hydroxyl radical (•OH) was used as radical target in aqueous medium, HAT results showed a negative Gibb's free energy value in about -39.0 kcal.mol^{-1}. The highly reactive oxidative free radicals (•R) are highly implicated in cancer progression. Free radicals (•R) could stimulate lipid peroxidation, oxidative damage to proteins, or induced DNA damage leading to fatal lesions in the cell that contribute to cancer initiation, promotion and progression. Based on DFT calculations, SAC-CAFA-MET and SAC-CAFA-PENT could be interrupting cell oxidative stress through a hydrogen atom transfer pathway (HAT), may be explaining their effects in modulation of ROS in SW480 cells.

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Table 2. Calculated reaction free energies (ΔG, kcal.mol^{-1}) under standard conditions in water for HAT, SPT-ET and SET-PT reaction channels between SAC-CAFA-MET, SAC-CAFA-PENT and selected ROS.

Against ·OH radical					
		SPT-ET		SET-PT	
	HAT	Step 1	Step 2	Step 1	Step 2
SAC-CAFA-MET	-39.0	49.6	-88.6	8.4	-47.4
SAC-CAFA-PENT	-39.1	51.7	-90.8	4.7	-43.8
Against $O_2^{\cdot-}$					
SAC-CAFA-MET	10.4	7.0	3.4	70.2	-59.8
SAC-CAFA-PENT	10.3	9.2	1.1	66.5	-56.2

In silico pharmacokinetic profile of promising SAC-CAFA-MET and SAC-CAFA-PENT

Early prediction of clinical pharmacokinetic indices is a valuable tool for the initial screening of potential therapeutic candidates to be used in cancer development. Herein, physiologically based pharmacokinetic (PBPK) modeling was used to predict eleven relevant biopharmaceutical properties to the success on cancer drug development [54], among them molecular weight, surface area (TPSA), $\log P_{o/w}$, the number of hydrogen bond donor, acceptor, human intestinal absorption and binding constant to human serum albumin. As listed in Table 3, we focus this study on the promising *SAC-CAFA-MET and* SAC-CAFA-PENT hybrids which displayed favorable pharmacokinetics indices with values comparable to 95% of known drugs (or recommended ranges for an ideal drug). According to the Lipinski's guidelines [55], SAC-CAFA-MET and SAC-CAFA-PENT hybrids have no violations of the criteria that would make them a likely orally active drug candidate in humans.

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Table 3. *In silico* physicochemical and drug-likeness properties for SAC-CAFA-MET and SAC-CAFA-PENT

Properties	SAC-CAFA-MET	SAC-CAFA-PENT
M.W [a]	337.390	393.497
PSA [b]	112.443	111.838
n-Rot Bond (<15)	9	13
n-ON[c]	5	5
n-OHNH[d]	3	3
Log $P_{o/w}$ [e]	2.694	4.175
Log K_{hsa} [f]	-0.002	0.403
App. Caco-2 [g] (nm/s)	166	219
App. MDCK[h] (nm/s)	97	134
% GI [i]	82	93
Lipinski Rule of five (≤1)	0	0

[a] Molecular weight of the molecule; [b] Polar surface area (PSA) (7.0–200.0 Å²); [c] n-ON number of hydrogen bond acceptors <10; [d] n-OHNH number of hydrogen bonds donors ≤5; [e] Predicted octanol–water partition coefficient (log $P_{o/w}$) (–2.0 to 6.5); [f] Logarithm of predicted binding constant to human serum albumin (log K_{hsa}) (-1.5 to 1.2); [g] Predicted human intestinal permeability model (App. Caco-2) (<25 poor, >500 great); [h]Apparent permeability across cellular membranes of Madin-Darby canine kidney (MDCK) cells (<25 poor, >500 great); [i] Human intestinal absorption (>80% is high, <25% is poor).

Regarding the degree of lipophilicity (expressed as log$P_{o/w}$), hybrids fit well within the ideal range for lipid-based formulations (–2.0 to 6.0) [56] with values of 2.694 and 4.175, respectively. Additionally, the top-two hybrids exhibited large numbers of human intestinal absorption (%GI) up to 82% reflecting that these compounds would have greater chance to be absorbed throughout the intestinal segments following oral administration. To contrast this parameter, permeation across traditional Caco-2 and MDCK cells models were used, which have been of great utility in prediction of intestinal drug absorption [57-59]. Thus, we founded that SAC-CAFA-MET and SAC-CAFA-PENT displayed significant permeability values in both Caco-2 and MDCK Caco-2/MDCK cells models (166 and 219 nm/s; 97 and 134 nm/s, respectively). We also computed Polar Surface Area (PSA) parameter that is strongly associated with the passive molecular transport through membranes and drug-membrane interactions. Thus, PSA values for the title hybrids displayed optimal therapeutic values of 112.443 and 111.838 $Å^2$, respectively, indicating that these compounds would penetrate deep into the tumor tissue. On the other hand, we also examined the drug–plasma protein binding (K_{HSA}) which is the major factor affecting drug distribution into the systemic circulation. For therapeutic uses, predicted logK_{HSA} profiling between -1.5 to 1.5 are recommended for potential drugs candidates [60,61]. SAC-CAFA-MET and SAC-CAFA-

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PENT exhibited affinities with HSA within the recommended range of -0.002 and 0.403 (expressed as $logK_{hsa}$). Altogether, merging S-allyl cysteine and caffeic acid sub-units into a unique structural core provide cytotoxic compounds with favorable pharmacokinetic profile, making this scaffold a valuable starting point for the development of novel agents in anti-cancer interventions. Combining experimental data and computational methods demonstrated that SAC-CAFA-MET and SAC-CAFA-PENT as promising antioxidant and caspase-targeting drug candidates towards colorectal cancer with optimal pharmacokinetic indices.

Conclusion

In summary, we have identified two hybrid molecules possessing potent chemopreventive activity in colorectal cancer. Some approaches to the possible mechanism of action of these molecules based on *S*-allyl Cysteine Ester - Caffeic Acid Amide, were made. Both compounds induced cell damage and reduce ROS formation, however, despite that both share a similar chemical nature, it was observed that they act through different mechanisms. Firstly, SAC-CAFA-PENT induced apoptosis mediated by caspase 3 but independent of p53, probably triggered by intrinsic route, with changes in mitochondrial membrane potential and a lack of activation of caspase-8. Besides, it was hypothesized that this hybrid induced anti-inflammatory effect mediated by down regulation of IL-6. In contrast, SAC-CAFA-MET induced cell death independent of caspases and p53, mediated by down regulation of BcL-2, besides, considering the lack of modulation of biomarkers related to extrinsic route, we hypothesized that this hybrid could induce the release of AIF from mitochondria, causing the loss of mitochondrial membrane potential and the canonical changes of cell death characterized by chromatin condensation, and DNA fragmentation. In addition, docking studies would suggest caspase-3 modulation as the primary mechanism by which hybrids elicit apoptosis in human colorectal adenocarcinoma SW480 cells. Meanwhile, DFT calculations suggest that hybrids would produce effects in modulation of ROS in SW480 cells via hydrogen atom transfer pathway (HAT). Finally, both, SAC-CAFA-MET and SAC-CAFA-PENT displayed a favorable pharmacokinetic profile. However, it is necessary to carry out further investigations to explore more deeply the potential benefits of these novel hybrids in different types of cancers.

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Materials and methods

In vitro biological assays

Cell line and culture medium

SW480 (human colon cancer) and CHO-K1 cells (Chinese hamster ovary, non-malignant) were obtained from The European Collection of Authenticated Cell Cultures (ECACC, England). Cells were grown in 25-cm^2 Falcon flasks containing Dulbecco's Modified Eagle Medium, supplemented with 10% heat-inactivated (56°C) horse serum, 1% non-essential amino acids and 1% penicillin/streptomycin procured from Gibco Invitrogen (Carlsbad, USA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 5 mg/ml transferrin, 5 ng/ml selenium and 10 mg/ml insulin (ITS-defined medium; Gibco, Invitrogen, Carlsbad, USA) [62-64].

Determination of ROS

Intracellular levels of ROS were determined as previously reported [65]. SW480 cells were seeded at a density of 2.5 x 10^5 cells/well in 6-well tissue culture plates allowing them to grow for 24 hours, afterwards, they were treated for 48 hours with either DMSO 1% (control) or hybrids SAC-CAFA-MET (124.2 µM) and SAC-CAFA-PENT (118 µM). CM-H2DCFDA was then added at a final concentration of 8 µM and incubated for 30 min at 37°C, protected from light. Analysis was made by flow cytometry. ROS production was expressed as percentage (%) increase in fluorescence relative to untreated control cells.

Determination of inflammatory cytokines and apoptotic proteins

Tumor cells (SW480) were cultured as previously described and then treated with hybrids SAC-CAFA-MET (124.2 µM) and SAC-CAFA-PENT (118 µM) for 48 hours. Afterwards, cells were collected by scraping and lysed with Cell Lysis Buffer (1X, Ref. #9803). The supernatant was used to determine the effect of the hybrids on the modulation of inflammatory cytokines, apoptotic biomarkers, and enzymes. The kits for detecting COX 1, COX2, IL-1β, IL6, TNFα were provided by Cayman Chemical Company (Ann Arbor, MI, USA); PGE2, caspase-7, caspase-8, MMP7 and MMP9 were obtained from Elabscience Biotechnology Co., (China); the determination of Bid was carried out through G-

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Biosciences, Inc. USA, whereas cleaved caspase-3, p53 and Bcl-2, were obtained from Cell-Signaling Technology (Danvers, Massachusetts, USA). The assays were performed according to manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times. Data are reported as mean ± SE (standard error). Statistical differences between control group (non-treated) and treated ones were evaluated by one-way ANOVA followed by the Dunnett's test. Values with $p \leq 0.05$ were considered significant. Data were analyzed with GraphPad Prism version 7.04 for Windows (Graph Pad Software, San Diego, California, USA).

Source of the hybrid molecules

All compounds were synthesized by the group "Química de Plantas Colombianas", Faculty of Exact and Natural Sciences from the University of Antioquia (Medellín, Colombia). These were characterized by spectroscopic techniques as infrared (IR) and nuclear magnetic resonance (NMR). The synthesis and cytotoxic activity of these hybrids was previously reported [17].

Computational methods

Chemical structure of hybrids **6**, including **SAC-CAFA-MET** and **SAC-CAFA-PENT**, as well as inhibitors Venetoclax and Ac-DEVD-CMK, were ligands used in these computational approaches. Their 2D structures were drawn in the ChemDraw 17.0 software (Cambridge Soft, USA) and saved as MDL MoL files. The Chem3D 17.0 (Cambridge Soft, USA) was used to generate 3D structures of all ligands and energetically minimize them by the MM2 force field. The discovery studies visualizer program was used to rewrite the data files into pdb format. AutodockTools were used to parameterized ligands structures through compute Gasteiger partial atomic charges and to add full hydrogens, as well as to assign rotatable bonds. Resulting structure was saved in the required format for use with AutoDock. Then, AUTOTUTORS in AutoDockTools was used to defined all possible flexible torsions of selected ligands to favor the computed binding with the receptor structure [66]. Caspase-3 (PDB ID: 5I9B), Bcl-2 (PDB: 4MAN) and IL-6 (PDB: 1ALU) crystal structures were

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downloaded from the Protein Data Bank (https://rcsb.org), and all bounded ligands, ions, and solvent molecules were manually removed using the DS Visualizer 2.5 program. For docking studies, the structure of selected proteins was parameterized using AutoDock Tools [67]. To facilitate the formation of hydrogen bonds, polar hydrogens were added. AutoDock Vina software was used to perform molecular docking and default procedures for docking a flexible ligand to a rigid protein. Then, ligands were centered at the binding site located into the binding cavity of the caspase-3, Bcl-2 and IL-6 proteins at x, y, and z coordinates of 1.5, -8.1and -13.4; -11.252, 13.128 and 5.816; -5.433, -12.86 and 0.496, respectively. In detail, docking studies involved a grid box which was identified by using Autodock Vina 1.1.2 and exhaustiveness was 20 for each protein-compound pair [67]. Catalytic active site was surrounded by a docking grid of 36 x 36 x 36 Å (for caspase-3), 32 x 32 x 32 Å (for Bcl-2) and 40 x 40 x 40 Å (for IL-6) with a grid spacing of 1Å. Ligand-binding affinities (in $kcal.mol^{-1}$) was estimated by AutoDock Vina and ranked based on the free-energy binding theory. Then, docking solutions were graphically inspected by using the DS Visualizer 2.5 (http://3dsbiovia.com/products/) to provide a 2D-ligand interaction plot, while ribbon surface representation of 3D model was explored by using The PyMOL Molecular Graphics System 2.0 programs.

On another hand, quantum calculations by means density functional theory (DFT) were performed to get a deeper insight of the relationship between **SAC-CAFA-MET** and **SAC-CAFA-PENT** hybrids structure and their antioxidant activity against ROS production in SW80 cells. The structures of all reactants and products were optimized using the Minnesota's M06-2X [68] DFT functional in conjunction with the 6-31++G(2d,p) basis set [68-72]. We choose this functional because it has shown to give good thermochemical results in reactions between phenolic-like compounds and free radicals [73-74]. All calculations were performed with the Gamess-US program [75]. Frequencies were calculated in order to characterize all minima stationary points (*e.g.* zero imaginary frequencies). Thermal correction, zero-point energies and entropy contributions were used to calculate Gibbs free energies at 298.15 K and 1 atm. In addition, aqueous media contribution to the Gibbs energy was included with the SMD (continuum solvation model) from single point calculations at the DFT optimized geometry in vacuum.

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Finally, eight pharmaceutical relevant properties were screened for active hybrids **SAC-CAFA-MET** and **SAC-CAFA-PENT** by combining several opensource cheminformatics toolkits such us Molinspiration software, ALOGPS 2.1 algorithm from the Virtual Computational Chemistry Laboratory and Pre-ADMET 2.0 program.

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Compliance with ethical standards: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest: The authors declare no competing interests.

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