Resistance modulatory and efflux-inhibitory activities of capsaicinoids and capsinoids

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ABSTRACT

Capsaicinoids are reported to have a bunch of promising pharmacological activities, among them antibacterial effects against various strains of bacteria. In this study the effect on efflux pumps of mycobacteria was investigated. The importance of efflux pumps, and the inhibition of these, is rising due to their involvement in antibiotic resistance development. In order to draw structure and activity relationships we tested natural and synthetical capsaicinoids as well as synthetical capsinoids. In an accumulation assay these compounds were evaluated for their ability to accumulate ethidium bromide into mycobacterial cells, a well-known substrate for efflux pumps. Capsaicin and dihydrocapsaicin, the two most abundant capsaicinoids in *Capsicum* species, proved to be superior efflux pump inhibitors compared to the standard verapamil. A dilution series showed dose dependency of both compounds. The compound class of less pungent capsinoids qualified for further investigation as antibacterials against *Mycobacterium smegmatis*.

1. Introduction

Capsicum species (plant family *Solanaceae*) are the source for pungent capsaicinoids, which are built up by the fusion of vanillin amine and branched fatty acids. Capsaicin (**1**) and dihydrocapsaicin (DHC, **2**) are the main representatives which together amount to over 90% of total capsaicinoids in *Capsicum* species (Fig. 1) [1]. The source of these pungent compounds can be traced back to the placental cells in *Capsicum* fruits. In total, more than ten varying capsaicinoids have been reported in literature. The corresponding capsinoids bear a vanillyl alcohol as starting point and are reported to be less pungent compared to capsaicinoids [2]. Capsiate and dihydrocapsiate are the major compounds in this chemical class [3].

Capsicum species are traditionally used as spices and to relieve pain conditions. The analgesic effect of capsaicin can be attributed to a desensitization of neurons. The binding of capsaicin (**1**) to the transient receptor potential vanilloid 1 (TRPV1) leads to a release of substance P which in turn stimulates C-fibres. This activation is responsible for the well-known burning sensation, pain and inflammation [4]. Heat and

pain receptors are activated and lead to an increased circulation which results in a pronounced removal of pain triggering substances. A prolonged usage of **1** entails desensitization by leaving neurons insensitive for physiological stimuli [5–7]. Compared to **1**, DHC (**2**) is a more potent agonist of TRPV1, which was shown by hypothermia experiments [8]. Additional to antioxidant, platelet aggregation inhibitory and anti-obesity effects, it is also reported that Capsicum extracts and **1** are active against various bacteria such as species of *Bacillus*, *Clostridium*, *Escherichia*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and *Streptococcus* [9–13]. Topical pharmaceuticals containing low doses of capsaicin are available as ointments and patches for the relief of muscle or joint pain, whereby a high dose capsaicin cream (8%) for single usage is under evaluation [14]. By using **1** topically, blood levels of around $10–20\,\mu M$ can be measured. Richeux et al. reported a half maximal inhibitory concentration of $175\,\mu M$ capsaicin when tested against human endothelial cells ECV 304 [15].

Due to the fact that bacteria rapidly adapt to environmental conditions, the number of resistant strains is rising worldwide. Multidrugand extensively drug-resistant bacteria challenge the global health
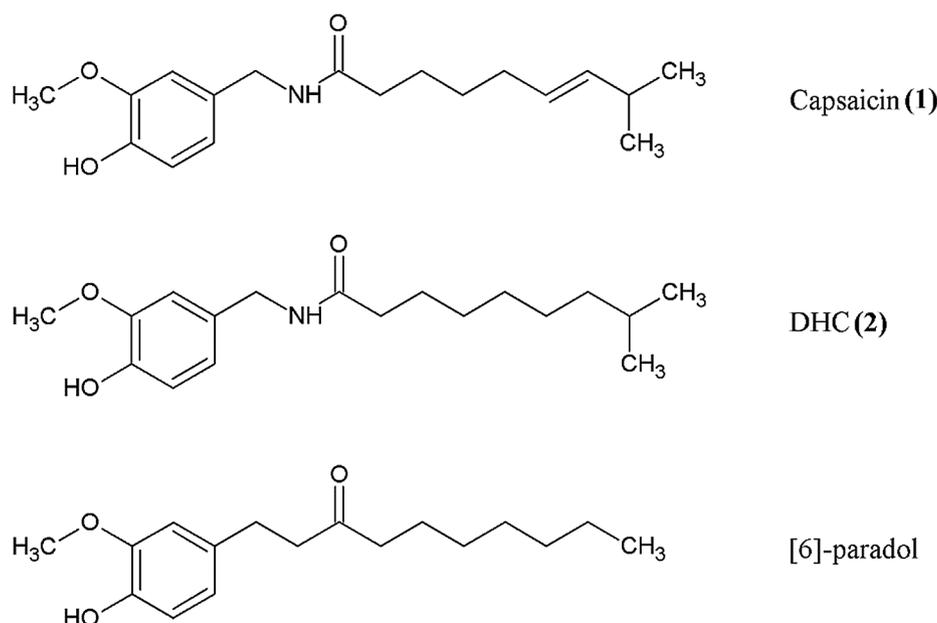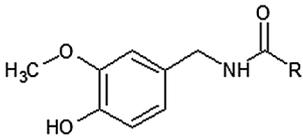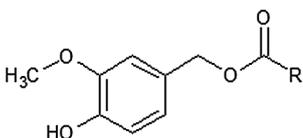
Fig. 1. Comparison of capsaicin (1) and DHC (2) and [6]-paradol.

Table 1
Detailed listing of capsaicinoid (3–16) and capsinoid structures (17–21).

R	Compound No.
-CH_3	3
-CH_2-CH_3	4
-(CH_2)_2-CH_3	5
-(CH_2)_3-CH_3	6
-(CH_2)_4-CH_3	7
-(CH_2)_5-CH_3	8
-(CH_2)_6-CH_3	9
-(CH_2)_7-CH_3	10
-(CH_2)_8-CH_3	11
-(CH_2)_9-CH_3	12
-(CH_2)_{10}-CH_3	13
-(CH_2)_{11}-CH_3	14
-(CH_2)_{12}-CH_3	15
-(CH_2)_{14}-CH_3	16
-(CH_2)_6-CH_3	17
-(CH_2)_7-CH_3	18
-(CH_2)_8-CH_3	19
-(CH_2)_9-CH_3	20
-(CH_2)_{10}-CH_3	21

system in the way that new therapeutics to overcome this resistant circle have to be developed on an urgent basis. One feasible way to combat this increase of resistance rates is the application of so called efflux pump inhibitors (EPI). Efflux pumps (EP) are ubiquitous expressed membrane transport proteins of bacterial cell walls which are able to extrude xenobiotics from the cytosol. With this outward transport of toxic compounds the bacterial cells secure their survival. When these EP are blocked, sufficient amounts of administered drug remain in the cell and therefore the therapeutic effect can be restored [16]. Therefore, these EPI represent a relatively new and efficient way of combating resistance in the new century. Plants have emphasized their role as pharmaceuticals and thus it is no surprise that many EPI have already been reported from various plants [17–20]. Additionally, an anti-cancer effect for 1 was reported in literature which is related to efflux. This effect can be traced back to the inhibition of p-glycoprotein by cutting the efflux of available cancer drugs in the cells [21]. Furthermore, an involvement in the up-regulation of tumor suppressor protein p53, responsible for cell cycle arrest and apoptosis, is discussed [4]. With these postulated effects, 1 was reported to have impact on

various human cancer cell lines [22]. For human lung carcinoma cells (A549) a half maximal effective concentration of 400 μM was determined for compounds 1 and 2 [4].

In order to increase the knowledge of putative EPI, capsaicinoids and their related lesser pungent capsinoids were investigated in this study and evaluated for their antibacterial, resistance-modulatory and efflux pump inhibitory effect in a mycobacterial model. In order to establish structure-activity relationships, particular attention was devoted to the influence of the fatty acid length on antimycobacterial and efflux inhibiting effects. The idea to evaluate capsaicinoids as putative EPI originated from the structural relation to [6]-paradol, which was found to be a promising EPI in one of our previous studies (Fig. 1) [18].

2. Material & methods

Capsaicinoids and capsinoids were synthetically manufactured as described below. Pure compound capsaicin (≥96%) was purchased from Sigma Aldrich and DHC (≥94%) from Phytolab.

2.1. Synthesis of capsaicinoids and capsinoids

Capsaicinoids (3–16) and capsinoids (17–21) were obtained by following the procedure described by Barbero and co-workers (Table 1) [23]. Purities of the compounds were determined by ^{1}H NMR and ^{13}C NMR analyses and found to be ≥ 98% in all cases. [1]H and ^{13}C spectra were recorded at room temperature using CDCl_3 as solvent, on Agilent INOVA spectrometer, at 399.572 and 100.460 MHz respectively. The resonance of residual chloroform was set to δ 7.25 ppm for ^{1}H and to δ 77.0 ppm for [13]C. These compounds were afforded with moderate-high yield.

2.2. Antibacterial testing

Minimal inhibitory concentration (MIC) and modulation factor (MF) were determined following published literature [24]. Surveying, extracts and pure compounds were screened against the non-pathogenic strain *Mycobacterium smegmatis* mc[2] 155 (ATCC 700084). The testing agents were diluted in dimethyl sulfoxide prior testing and added to 96 well plates together with Mueller Hinton Broth. In the final assay solution dimethyl sulfoxide concentration did not exceed 3.6%. For control purposes, isoniazid was integrated on each plate together with the

additional sterile and growth controls. At concentrations corresponding to half of their MIC, compounds were examined for their modulatory activities on the MIC of ethidium bromide (EtBr) and rifampicin (RIF), respectively. For the determination of the MF, the concentration of pure compounds was kept constant at $^1/_2$ MIC and EtBr or RIF were serially diluted over the plate. Incubation was done with a 5×10^5 colony forming units (CFU/ml) bacterial suspension for 72 h at 37 °C. Analysis was carried out using the visual colour change of MTT (thiazolyl blue tetrazolium bromide, Sigma Aldrich). The MF was used to express the modulating effects on the MIC of EtBr or RIF. It was calculated according to the following formula: MF = (MIC antibiotic)/(MIC antibiotic + modulator). Selection criteria for further investigation were a MF_{EtBr} of ≥ 4 or $MF_{RIF} \geq 8$. If these criteria were met, the MF assay was performed vice versa using a constant concentration of antibiotic in order to evaluate the fractional inhibitory concentration index (FICI). By comparing both results the FICI was determined which provides insight into synergistic effects evoked by the tested compound. Synergy is given when FICI values are below 0.5 [25]. In total, 16 capsaicinoids (14 synthetical and 2 natural) and 5 capsinoids underwent detailed investigation.

2.3. Accumulation assay

If screening results were promising, pure compounds were driven to the accumulation assay which determines the potential of substances to accumulate EtBr, a substrate of many EP, into bacterial cells. The outcome, compared to standards, is a prediction marker for the efflux pump inhibitory activity. Compounds were prepared in subinhibitory concentrations ($^1/_2$ MIC) together with 0.5 mg/L EtBr and 0.4% glucose. This solution was added to a plate in equal amounts with a bacterial inoculum adjusted to an optical density of 0.4 containing Tween 80. Standard EPI (verapamil (VP), chlorpromazine (CP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and phenylalanyl-arginyl-beta-naphthylamide (PAßN)) were used as comparative probes. The fluorescent increase was measured using a Wallac 1420 Victor2™ multilabel counter. Measurement time points were collected every 50 s for 60 min total time at 37 °C using an excitation wavelength of 531 nm and an emission wavelength of 590 nm. Each experiment was carried out in triplicate and summarized in one graph. If the obtained results were superior compared to VP, additional assays with a dilution series down to $^1/_{32}$ MIC were performed to check the presence of concentration-dependent effects.

3. Results & discussion

3.1. Antimycobacterial effect

First, two pure compounds **1** and **2** found in *Capsicum* sp. (Fig. 1), fourteen synthetically produced capsaicinoids (**3–16**) as well as five capsinoids (**17–21**) were evaluated for their antimycobacterial potential against *M. smegmatis* with special attention drawn to the chain length.

From Table 2 it can be clearly seen, that the effect evoked from capsaicinoids is of resistance-modulatory nature, however, no antimycobacterial potential was detected against *M. smegmatis* due to the obtained high MIC of > 128 mg/L. This resistance-modifying effect of capsaicinoids can be traced back to a certain length of the side chain. In case of side chains bearing 7 to 11 C-atoms a promising resistance modulation is given. For capsaicinoids with $MF_{EtBr} \geq 4$ detailed investigation was carried out including an additional MF with RIF and the accumulation assay using EtBr. When tested with RIF, no noteworthy modulatory effect could be detected against mycobacteria and therefore no evaluation of FICI was carried out. The naturally found capsaicinoids, **1** and **2** coincided with the absence of antimycobacterial activity but the obtained MF varied from synthetically ones. The most promising capsaicinoid in terms of resistance modulation of EtBr was **2**

Table 2
Antimycobacterial activities of capsaicinoids and capsinoids.

Compound	MIC (mg/L)	MF_{EtBr}	MF_{RIF}
Capsaicin (**1**)	> 128	**4**	1
DHC (**2**)	> 128	**32**	**8**
3	> 128	1	–
4	> 128	1	–
5	> 128	1	–
6	> 128	1	–
7	> 128	1	–
8	> 128	1	–
9	> 128	**4**	1
10	> 128	**8**	2
11	> 128	**8**	2
12	> 128	**4**	2
13	> 128	**8**	2
14	> 128	2	–
15	> 128	2	–
16	> 128	1	–
17	> 128	2	–
18	> 128	2	–
19	> 128	2	–
20	64	2	–
21	32	2	–

MIC: minimal inhibitory concentration, MF: modulation factor, MF = (MIC antibiotic)/(MIC antibiotic + modulator), EtBr: ethidium bromide, RIF: rifampicin, -: not tested.

with a MF of 32, i.e. a 32 times lower amount of antibiotic is needed to evoke the same effect [26]. An additional evaluation against RIF gave rise to a MF of 8 which in turn is the cut-off for FICI evaluation. Therefore, both set-ups of MF assay were performed and by comparing the results a synergy between **2** and RIF was detected with a FICI value of 0.38. Structural similarity can be drawn between natural capsaicinoids and capsaicinoid **11**. All of these compounds have a C9 side chain in common whereas the natural capsaicinoids bear a branched chain end. It can be concluded that branching of the side chain leads to an increase of modulatory power in case of **2** (MF_{EtBr} of 32) whereas compound **11** only revealed a MF_{EtBr} of 8. The introduction of a double bond in this side chain, as found in **1**, further decreases the modulatory activity to a factor of 4. From the obtained results only those capsinoids with corresponding chain lengths of the most active capsaicinoids were evaluated for their antimycobacterial and resistance-modulatory effect. These five synthetically produced capsinoids demonstrated that the higher the number of C-atoms in the side chain, the better the antimycobacterial effect. Capsinoid **21** was able to inhibit *M. smegmatis* with a MIC of 32 mg/L and was the most active capsinoid carrying a side chain equipped with 11 C-atoms. Capsinoids exerted only weak modulatory power with a MF_{EtBr} of 2 and were therefore not further investigated. Nitrogen substituted side chains are able to exert a higher resistance modulatory power, whereas oxygen substitution in the side chains leads to higher antimycobacterial effects with lower MIC values.

3.2. EtBr accumulation

To evaluate the potential of accumulating EtBr into mycobacterial cells, the accumulation assay was performed with these pungent components at $^1/_2$ MIC concentration. Due to the promising modulatory effects of derivatives **9–13** these compounds were included in the testing together with the two natural capsaicinoids (**1** and **2**). This assay takes advantage of the fact that EtBr emits a strong fluorescent signal when bound to intracellular components and only has a weak signal when present extracellularly [27]. Fig. 2A shows the accumulation behaviour of natural capsaicinoids confronted to standard EPI whereas VP (red) constitutes the best standard EPI. **1** as well as **2** were evaluated as superior EPI than VP whereas **2** was the best putative EPI candidate of all tested compounds with an additional effect of 22.9% compared to
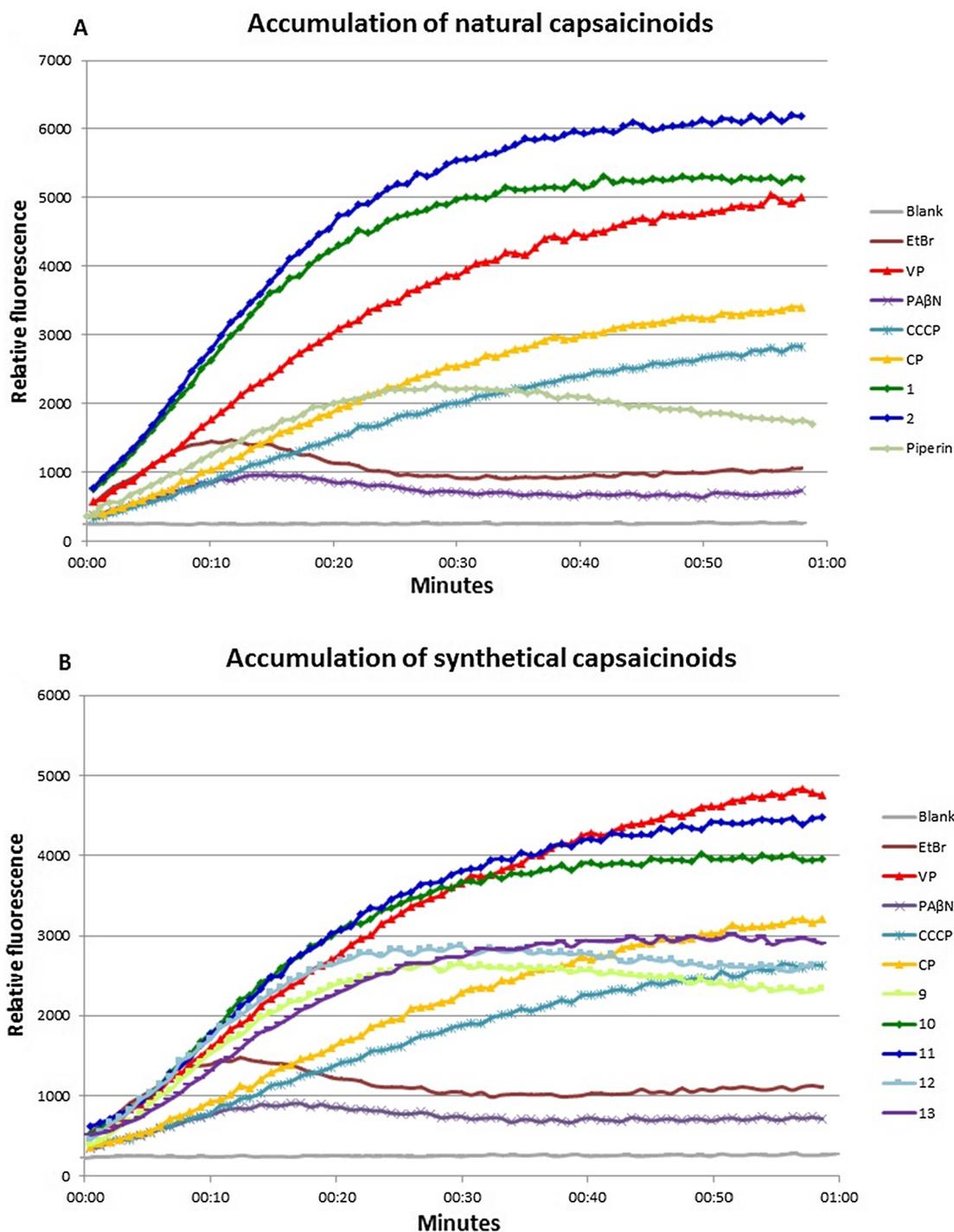
Fig. 2. EtBr accumulation of natural capsaicinoids (A) and synthetical capsaicinoids (B) EtBr: ethidium bromide, VP: verapamil, PAβN: phenylalanyl-arginyl-beta-naphthylamide, CCCP: carbonyl cyanide m-chlorophenyl hydrazine, CP: chlorpromazine.

VP (measured at 60 min). Piperine, an alkaloid isolated from *Piper nigrum* and dedicated as EPI [28], was included in this first evaluation to see how other pungent compounds act in this environment. We could not confirm the postulated EPI activity of piperine in our experiment due to a measured effect below the standard CCCP. Although all synthetical capsaicinoids were below the effect of VP, but still superior compared to CP, we decided to undergo detailed investigation with compounds **10** and **11**, which in turn could prove helpful to draw structure activity correlations. Therefore, additional accumulation experiments with capsaicin (**1**), DHC (**2**), **10** and **11** were performed by

testing serial dilutions down to $^1/_{32}$ MIC (128 mg/L–8 mg/L) (see Fig. 3).

The best putative EPI candidate DHC (**2**) showed a definite superior capacity of accumulating EtBr into the cells of *M. smegmatis*. Test concentrations of $^1/_2$ MIC and $^1/_4$ MIC were better or equal to VP, respectively, whereas both slopes were identical until minute 15. Concentrations of $^1/_8$–$^1/_{32}$ MIC of all four tested substances showed lower activity compared to CP but performed better than the EtBr control. It could be shown in all cases that the accumulation behaviour was concentration-dependent which leads to the assumption that the

effect is specific to EP. Capsaicin (**1**), the unsaturated counterpart of DHC (**2**), showed a decreased accumulation potential whereas 128 mg/ L concentration ($^1/_2$ MIC) was still above the effect of VP. A concentration of 64 mg/L ($^1/_4$ MIC) resulted in an accumulation behaviour located between VP and CP. Synthetical capsaicinoids displayed a slightly variable accumulation behaviour. Not VP but CP was the accumulation indicator due to the fact that these syntheticals did not reach the effect of VP in the overview experiment. Both compounds are only distinguished by an additional methyl group in the side chain. Concentrations of $^1/_2$ and $^1/_4$ MIC of **11** were located above the effect of CP. In the case of **10** only the $^1/_2$ MIC concentration was able to accumulate more EtBr within the cells compared to CP. 64 mg/L of **10** ($^1/_4$ MIC) was slightly below the effect of CP. Due to the lower EtBr

accumulation power of **10** (C8 side chain) it can be concluded that capsaicinoids bearing a C9 side chain are preferable when considering the efflux-pump inhibitory activity.

4. Conclusions

Pungent capsaicinoids from *Capsicum* species were evaluated as putative EPI against mycobacteria. DHC (**2**) was found to be best candidate in this study, but also capsaicin (**1**) was above the EPI value of the standard verapamil. The dose-dependent effect of **2** exceeded the value of VP by about 23% (at 60 min). EPI isolated from *Aframomum melegueta,* i.e. [6]-paradol and [8]-gingerol, are comparable with capsaicinoids by means of chemical structure but do not elicit the same
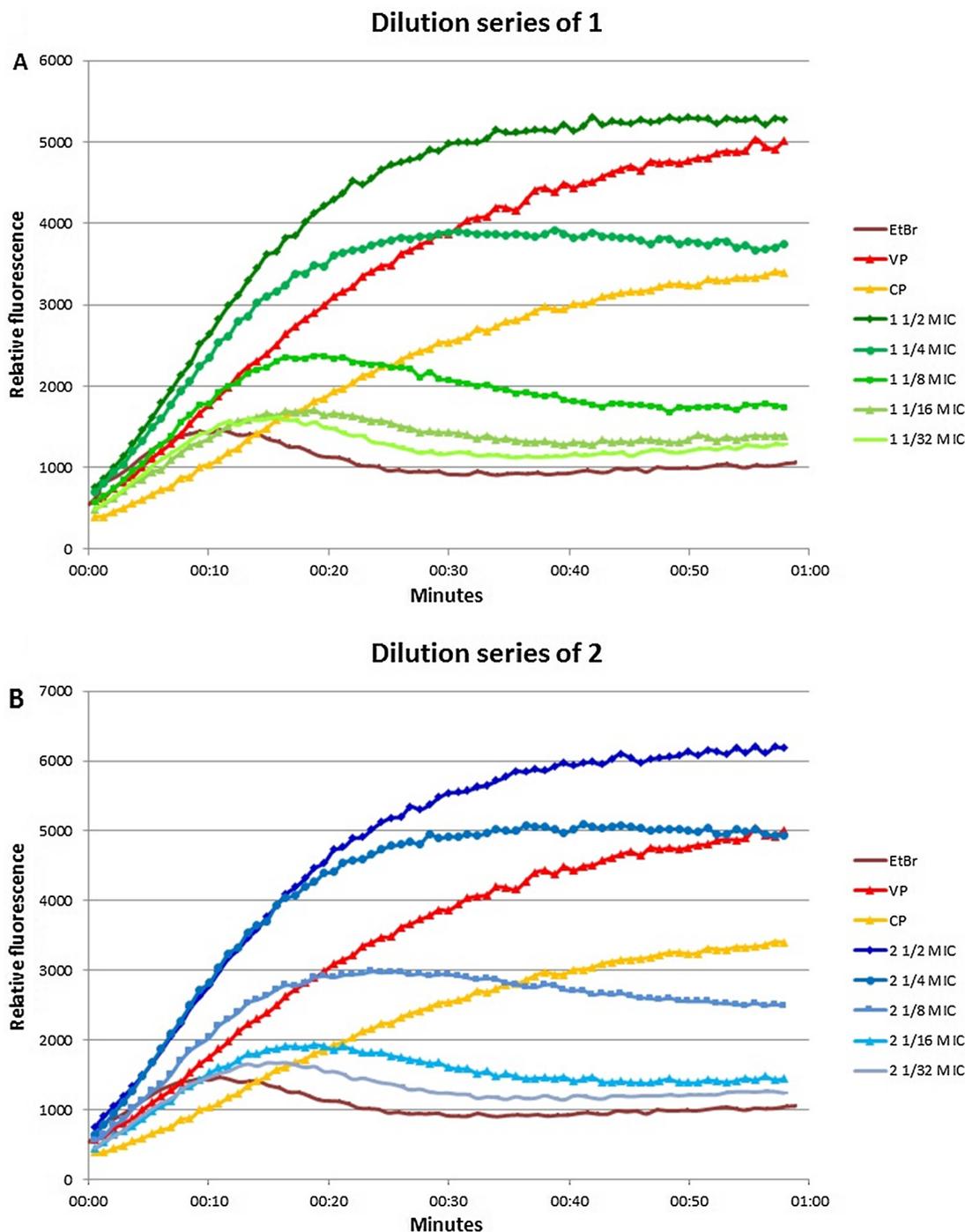

Fig. 3. Serial dilution accumulation experiments of capsaicin (**1**; A), DHC (**2**; B), **10** (C) and **11** (D) EtBr: ethidium bromide, VP: verapamil, CP: chlorpromazine.
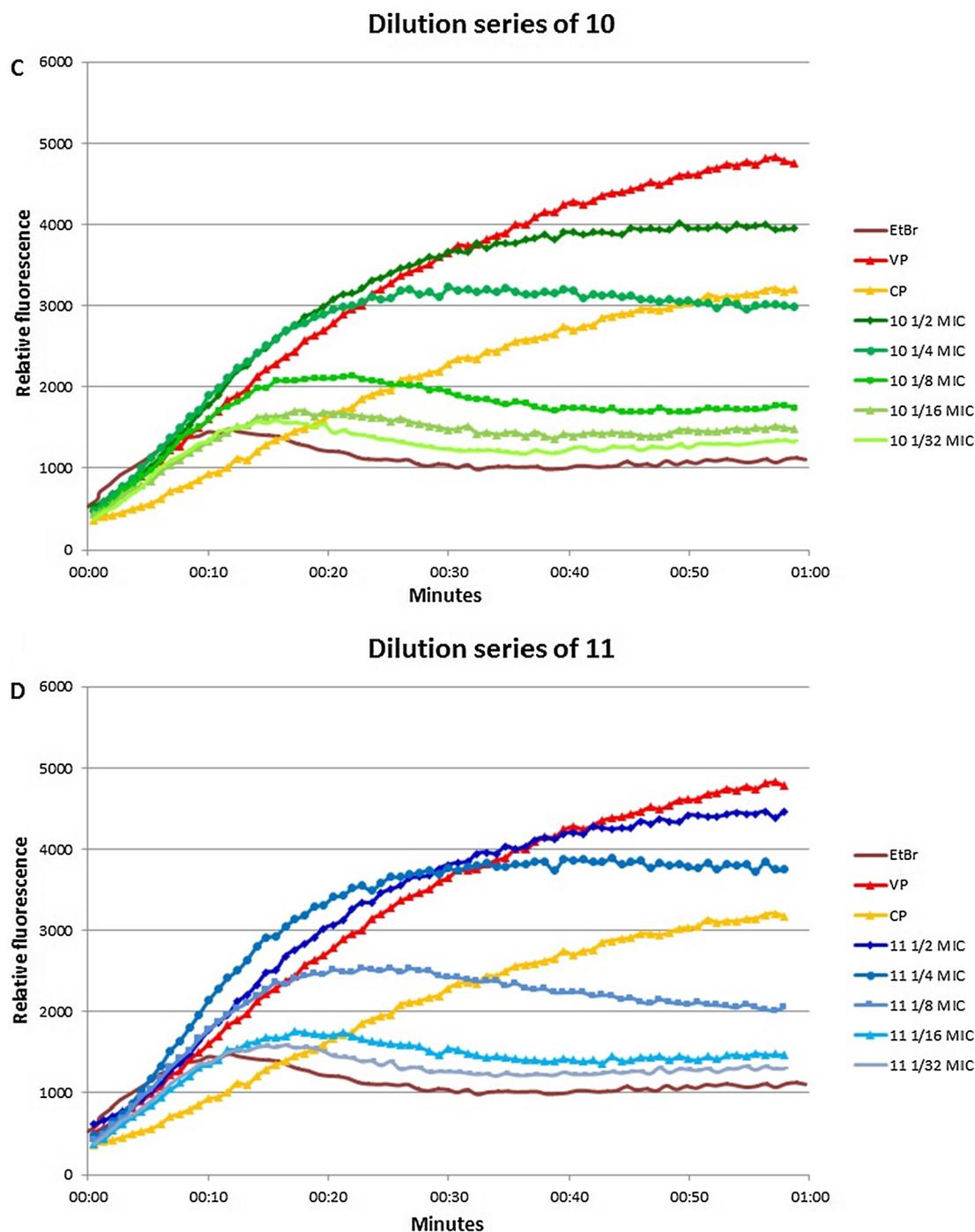
Dilution series of 10

Dilution series of 11

Fig. 3. (*continued*)

promising EPI effect and showed a lower EtBr accumulation potential compared to VP. By comparing natural capsaicinoids with synthetical capsaicinoids and synthetical capsinoids (O-substitution in the side chain) we were able to get insight into structural relationships. In case of synthetical capsaicinoids, the antimycobacterial effect was in accordance with the naturally derived substances. In regard of the resistance modulatory activity, a side chain length of 7 to 11 carbon atoms seems to be preferable, whereas this effect did not exceed the one of compound **2**. In case of synthetical capsinoids the longer the side chain the better was the detected antimycobacterial effect. Therefore, capsinoids bearing a longer side chain than 11 C-atoms need to be evaluated for their antibacterial effect against mycobacteria. In conclusion, capsaicinoids equipped with a branched and saturated C9-side chain are ideal for promising resistance modulatory and efflux-pump inhibitory activities. As can be seen in case of **1**, the unsaturation of the fatty acid side chain is detrimental for both effects. The difference of just one methyl-group in case of **10** and **11** demonstrates the high sensitivity of mycobacterial efflux pumps to chemical variable structures.

Deeper investigations of DHC (**2**) as EPI need to be conducted in order to gain more insight into the mode of action of this capsaicinoid. Furthermore, the detected antimycobacterial effects give hope that non-pungent capsinoids with increased chain length can be developed as antibacterials.

Conflicts of interest

None.

Acknowledgements

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