Voltage operated potassium (Kv) channels contribute to endothelium-dependent vasorelaxation of carvacrol on rat aorta.

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Abstract

Carvacrol, a monoterpene widely present in nature, is commonly used in the food industry and in cosmetics, besides to possess a plethora of pharmacological properties; among these also *in vitro* vasorelaxing effects and *in vivo* hypothensive responses.

Although in rat aortic rings carvacrol evoked a vasodilatation both in the presence and in the absence of endothelium, in preparations with intact endothelial layer its vasoactive response appeared markedly improvement.

Objectives- this study aimed at investigating the mechanism of action responsible for the endothelial component of the carvacrol-induced vasorelaxing response observed rat isolated aortic rings.

Key findings- Pharmacological characterization led us to exclude the involvement of NO-pathway (neither L-NAME, NO-biosynthesis inhibitor, or ODQ, guanylate cyclase inhibitor, were able to modify the vascular effects of carvacrol) and of arachidonic acid cascade (no inhibitor intercepting the cascade influenced the endothelial-dependent vasodilatation of the monoterpene). Moreover, endothelial TRP channels weren't also involved, since capsazepine was any effect.

Finally, endothelial potassium channels were considered as possible targets of carvacrol; indeed, two voltage-operated potassium (Kv) channel blockers, 4-aminopyridine and quinine significantly reduced carvacrol potency and efficacy indices.

Conclusions- Kv channels seems to be responsible for vascular effects of the monoterpene typical of Labiatae family.

Keywords: carvacrol, essential oils, endothelial potassium channels, vasorelaxation, voltage-operated potassium channels.

Introduction

Carvacrol, a monoterpene widely present in nature, is the major constituent of the essential oils from various aromatic plants, especially of Labiatae family, such as Thymus vulgaris, Origanum compactum and Satureja montana. Carvacrol is commonly used in the food industry and in cosmetics, as preservative and antioxidant. Interestingly, it possesses also a myriad of pharmacological properties, such anti-inflammatory, antibacterial, antitumor and antiplatelet ones (1-4).

Moreover, this monoterpene is reported to exhibit vasorelaxing activity on rat isolated aorta (5) and more recently on rat mesenteric arteries (6). Indeed, when administered i.p. to normotensive rats, carvacrol induced hypotensive responses (7). Although Peixoto-Neves et al. hypothesized the involvement of calcium channels in the concentration-dependent reduction of KCl- or phenylephrine-induced contractions, presently no clear mechanism of action has been assigned.

On the other hand, carvacrol is known also as agonist of transient receptor potential (TRP) cation channels, in particular TRPA1 and TRPV3 (8). Interestingly, Earely et al. demonstrated that carvacrol concentration-dependently activates calcium influx, produces endothelium-dependent vasodilatation and stimulates TRPV3 current in endothelial cells, isolated from cerebral and cerebellar arteries (9) .

Although available data on vascular effects of the monoterpene, to date underlying mechanisms the vasorelaxation mediated by carvacrol aren't again fully understood; particularly this study aimed at investigating the mechanism of action responsible for the endothelial component of the carvacrol-induced vasorelaxing response observed rat isolated aortic rings.

Material and methods

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609 and have been approved by the Ethical Committee for animal experimentation of Pisa University. To determine a possible vasodilatory mechanism of action, carvacrol was tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). The rats were sacrificed by an overdose of sodium pentobarbital. The aortae were immediately excised and freed of extraneous tissues. Five millimeters wide aortic rings were suspended, under a preload of 2g, in 20 ml organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$11.9; glucose 5.5), thermostated at 37°C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%).

Changes in tension were recorded by means of an isometric transducer (Grass FTO3 Basile mod. 7005), connected with a preamplifier (Buxco Electronics) and with a software of data acquisition (BIOPAC Systems Inc., MP 100). After an equilibration period of 60 min, the endothelium preservation or removal was confirmed by the administration of acetylcholine (ACh; 10 µM) to KCl 20mM-precontracted vascular rings. Preparations were considered to possess an intact endothelium, when the vasorelaxant response to Ach was greater than 75% of the KCl-induced contraction. When required from experiment procedures, the endothelium was removed immediately after dissection by gentle rubbing of the aortic lumen with a hypodermic needle. A Ach-induced relaxation <5% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer; while the organs, showing a relaxation between 5-75 % were discarded.

About 30–40 min after the endothelium evaluation, the aortic preparations were contracted by KCl 20mM and when the contraction reached a stable plateau, three-fold increasing concentrations of carvacrol (0.1 µM–100µM) were added cumulatively. Preliminary experiments showed that KCl 20mM-induced contractions remained in a stable tonic state for at least 60 min.

Several sets of experiments have been carried out on rat aortae, as reported in the following experimental protocols.

Experimental Protocol 1 –Absence of endothelium.

Aortic rings denuded from endothelium layer were contracted by KCl 20mM or KCl 60mM and a cumulative concentration-dependent curve was constructed by the addiction of carvacrol (0.1 µM–100µM)

Experimental Protocol 2 –Presence of endothelium/Evaluation of NO pathway.

Aortic rings were incubated with L-NAME 40 µM (NO biosynthesis inhibitor), or ODQ 1 µM (guanylate cyclase inhibitor) or the respective vehicles (bidistilled water and ethanol) for 20 minutes, then KCl 20mM was administered to evoke the contraction and finally a cumulative concentration-dependent curve was constructed by the addition of carvacrol (0.1 µM–100µM).

Experimental Protocol 3 –Presence of endothelium/Evaluation of arachidonic acid cascade.

In order to evaluate the involvement of arachidonic acid cascade, aristolochic acid 100 µM (A.A., phospholipase A_2 inhibitor), indomethacin 1 µM (cyclooxygenase inhibitor), baicalein 10 µM (lipoxygenase inhibitor) or miconazole 10 µM (citrochrome P450 inhibitor) were incubated 20 minutes before KCl 20mM-induced contraction and then a cumulative concentration-response curve was obtained adding carvacrol (0.1 µM–100µM).

Experimental Protocol 4- Presence of endothelium/Evaluation of vanilloid receptors.

In order to evaluate the involvement of endothelial vanilloid receptors, capsazepine 10 µM (TRPV1 inhibitor) was incubated 20 minutes before KCl 20mM-induced contraction and then a cumulative concentration-response curve was obtained adding carvacrol (0.1 µM–100µM).

Experimental Protocol 5-Presence of endothelium/Evaluation of potassium channels.

In order to evaluate the involvement of potassium channels, cumulative concentration-response curve was obtained adding carvacrol (0.1 µM–100µM) on rat aortic rings precontracted with KCl 60mM, a such experimental approach is used to evaluate the involvement of potassium channels. Moreover, in order to find the potassium channel involved in the action mechanism of carvacrol, tetraethylammonium chloride 10 mM (TEA, blocker of different subtypes of voltage-activated potassium channels), glibenclamide 1 µM (blocker of ATP-sensitive potassium channels), 4-aminopyridine 3mM (4-AP, blocker of many classes of voltage-operated potassium channel), apamin 250 nM (small calcium-activated potassium channel (SKCa) blocker), charibdotoxin 100nM (big- and intermediate-calcium-activated potassium channel (BKCa/IKCa) blocker) or iberiotoxin 100 nM (selective BKCa blocker) were added after the achievement of a steady contraction evoked by KCl 20mM (eventual increase was expressed as a percentage of the KCl-evoked contractile response). After achieving a stable plateau, cumulative concentration-response was obtained adding carvacrol (0.1 µM–100µM).

Data analysis

The vasorelaxing efficacy of carvacrol was evaluated as the maximal vasodilatory response (Emax), evoked by the highest concentration (100µM), expressed as a % of the contractile tone induced by KCl 20mM; while the parameter of potency was expressed as pIC50, calculated as the negative Logarithm of the molar concentration of carvacrol, evoking a half reduction of the contractile tone.

The parameters of efficacy and potency were expressed as mean ± standard error, for 6–10 experiments. ANOVA was selected as statistical analyses, moreover individual differences between treatments were evaluated using a post hoc test of Bonferroni. $P<0.05$ was considered representative of a significant statistical difference. Experimental data were analysed by a computer fitting procedure (software GraphPad Prism 5.0).

Drugs

Carvacrol was purchased from Fluka. All drugs used for investigation of vasorelaxing mechanism of carvacrol, acetylcholine and KCl were purchased from Sigma-Aldrich.

All solutions were freshly diluted immediately before the pharmacological experimental procedures from stock solutions using opportune solvents and stored at -20°C.

Previous experiments showed a complete ineffectiveness of the vehicles.

Results

Carvacrol produced full vasorelaxing effect on KCl 20mM-precontracted rat aortic preparations, both in the presence and in absence of the endothelial layer, although the potency index (pIC50) was markedly increased in aortae with endothelium (6.0±0.04) if compared to without it (5.2±0.03) (Figure 1).

Role of NO-pathway

The NO-biosynthesis inhibitor L-NAME 40 μM, and the guanylate cyclase inhibitor, ODQ 1 μM, didn't antagonize carvacrol-induced vasorelaxant response (Figure 2a; Tab 1).

Role of arachidonic acid pathway

Aristolochic acid 100 microM, a phospholipase A2 inhibitor, didn't significantly antagonize carvacrol-induced vasorelaxing effect (Figure 2b). Likely, neither the cyclooxygenase inhibitor indomethacin 1 μM, nor the lipoxygenase inhibitor baicalein (10 μM), nor miconazole 10 μM, a

cytochrome P450 inhibitor, significantly influenced the vasorelaxing response of carvacrol on aortic rings (Figure 2b; Tab. 1).

Role of TRP receptors

Capsazepine, a non-selective TRP antagonist, 10 μM didn't antagonize vasorelaxing effect of carvacrol (Figure 2c, Tab.1).

Role of potassium channels

In aortic rings without endothelium pre-contracted with KCl 60 mM, carvacrol-induced vasorelaxation wasn't significantly influenced (Figure 3b), suggesting that in these experimental conditions potassium channels aren't engaged. On the other hand, in presence of endothelium, the vasorelaxing effect of carvacrol was markedly reduced on aortae pre-contracted with KCl 60mM, indeed both pIC50 and efficacy were significantly decreased, suggesting a pivotal role of endothelium potassium channels (Tab.1, Figure 3a).

-Involvement of SKCa/IKCa/BKCa channels

Neither TEA 10 mM, nor iberiotoxin 100nM nor charibdotoxin 100nM nor apamine 250nM significantly antagonized the vascular effects of the monoterpene (Figure 4a).

-Involvement of ATP –sensitive potassium channels

KATP channels didn't seem involved in the endothelium-dependent vasorelaxing effects of carvacrol, since glibenclamide 10μM didn't modify its vascular action (Figure 4b , Tab.1).

-Involvement of voltage-gated potassium channels.

4-AP 3mM, a non-selective Kv blocker, markedly antagonized the endothelium-mediated vasorelaxing response of carvacrol, reducing its potency index to 4.8±0.08 (Figure 4c, Tab. 1).

Further, another non-selective blocker of Kv channels, indeed quinine 200 μM also significantly antagonized the carvacrol action.

Discussion

Carvacrol, a monoterpene of natural origin, induces vasorelaxation in isolated vessels from rat, observed on mesenteric arteries, mainly through an inhibition of calcium-influx, with a participation of the TRP channels (6). Previous studies on smooth muscle and vascular beds also reported vasoactive profile for this monoterpene, as well as a dose-dependent hypotension and bradycardia on anaesthetized rats (5,7).

In this paper, our attention was focused on the endothelium-dependent vascular effect of the monoterpene; indeed in the preparations with intact endothelium, carvacrol produced more potent vasorelaxation on the KCl20mM-precontracted aortae that in preparations without endothelium (Fig 1; Tab1); suggesting a relevant contribute of some mediators of endothelial origin in its vascular effects. Indeed, endothelial cells regulate vascular tone by releasing various contracting and relaxing factors including nitric oxide (NO), arachidonic acid metabolites, reactive oxygen species and vasoactive peptides (10). It is well-known that NO-pathway plays a pivotal role in the homeostasis of vascular tone, nonetheless in our set of experiments treatment with L-NAME or ODQ didn't modify vascular profile of the monoterpene (Fig. 2a, tab.1), leading to hypothesize that carvacrol-mediated endothelial mechanism is independent from NO biosynthesis or release. This result is in agreement with previous observations of Peixoto-Neves et al. (5).

On the other hand, arachidonic acid can be a precursor at endothelial level of several vasoactive mediators playing a critical role in the cardiovascular functions. Particularly, it can be metabolized by means of cyclooxygenase enzyme in order to produce prostacyclin (PGI2), by means lipoxygenase can be synthetized hydroperoxytetraenoic acids (HPETE), precursors of leucotriens and by means cytochrome P450 monooxygenase can be obtained epoxyeicosatrienoic derivatives (11, 12, 13).

Nevertheless, aristolochic acid (100 μM), an inhibitor of phospholipase A2 that prevents the arachidonic acid mobilization from cell membrane, didn't antagonize vascular effect of carvacrol; similarly to indomethacin (1 μM), an inhibitor of cyclooxygenase enzyme, baicalein (10 μM) a flavonoid endowed with lipoxygenase inhibitor activity and miconazole (10 μM) an anti-mycotic derivative used to block cytochrome P450 (Fig.2b, tab.1), suggesting that arachidonic cascade was not involved in the endothelial response of carvacrol.

Recently, the participation of TRP channels has been demonstrated on mesenteric arteries, however, the subtype of TRP channel involved is yet missing.

At endothelium level have been described several subtype of TRP channels, in particular of vanilloid subtypes, TRPV1, TRPV2, TRPV3 (14, 15). In this study capsazepine, a non-selective antagonist that has been used in order to evaluate the role of endothelial vanilloid receptors, didn't inhibit the carvacrol-induced vasorelaxing response; leading to speculate that in these experimental conditions, unlikely to mesenteric arteries (6), TRP channel has a marginal role.

Vascular function can be also regulated through several type of potassium channels, expressed on sarcolemmal but also on endothelial layer. On this regard the presence of calcium-dependent potassium channel endowed with different grade of conduction, big (BKCa), intermediate (IKCa) and small (SKCa), ATP-sensitive (KATP) and voltage-operated potassium channels (Kv) have been widely reported (16).

The involvement of potassium channels has been confirmed using KCl60mM to induce vasocontraction; indeed, a high level of depolarisation (induced by KCl60mM) is expected to induce a dramatic reduction of the parameters of potency and efficacy of drugs activating potassium channels (17) (Fig. 3, tab.1). Unlikely, in aortic preparations deprived of endothelium layer high membrane depolarization didn't influence neither potency nor efficacy of carvacrol,

suggesting that potassium channels are involved only in the endothelium-dependent vasodilatation of monoterpene.

Calcium-activated potassium channels are discarded, indeed selective and non-selective blockers were without effect on vascular action of carvacrol (Fig. 4a, tab.1), similarly, carvacrol didn't open endothelial KATP channels (Fig. 4b, tab.1).

Finally, voltage-operated one seemed to be the responsible for vasoactive profile of carvacrol, as demonstrated by using 4-AP and quinine, both Kv inhibitors (Fig. 4c, tab.1).

Concluding, endothelium-dependent action mechanism through which carvacrol produces a vasorelaxation on in vitro preparations of rat aortic rings is related to the engagement of voltage-operated potassium channels; indeed their opening provoke an hyperpolarization with consequent relaxation of aortic smooth muscle.

Conflict to interest

The authors report no conflicts of interest.

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Legends

Table 1 Potency and Efficacy indexes of carvacrol obtained pre-treating aortic preparations with intact endothelial layer.

Figure 1. Concentration-dependent curve obtained with carvacrol in the presence and in the absence of endothelium on rat aortic rings.

Figure 2. Concentration-dependent curve obtained with carvacrol, **a)** in the presence and in the absence of NO-pathway inhibitors (L-NAME and ODQ); **b)** in the presence and in the absence of

arachidonic acid cascade (Aristolochic acid, Indometacin, Baicalein, Miconazole); **c)** in the presence and in the absence of Capsazepine.

Figure 3. Concentration-dependent curve obtained with carvacrol, **a)** on aortic rings with intact endothelium pre-contracted by KCl 20mM or KCl 60mM; **b)** on aortic rings without endothelium pre-contracted by KCl 20mM or KCl 60 mM.

Figure 4. Concentration-dependent curve obtained with carvacrol, **a)** in the presence and in the absence of calcium-activated potassium channel blockers (TEA, ChTX, IbTX and Apamin); **b)** in the presence and in the absence of ATP-sensitive potassium channel blockers (Glibenclamide); **c)** in the presence and in the absence of voltage-operated potassium channel blockers (4-AP and quinine).

Table 1

Pharmacological treatment (*)	**pIC50 (M)**	**Efficacy (%)**
Vehicle (without Endo)	5.20±0.03	full
KCl60mM-contraction (without Endo)	4.90±0.1	full
Vehicle	6.00±0.04	93±2
KCl60mM-contraction	4.99±0.03	95±2
L-NAME 40 µM	5.97±0.10	Full
ODQ 1µM	5.74±0.08	full
A.A. 100 µM	5.71±0.10	92±12
Indometacin 1µM	5.72±0.06	98±2
Baicalein 10µM	5.61±0.09	full
Miconazole 10µM	5.44±0.15	85±15
TEA 10mM	6.20±0.15	93±8
Caribdotoxin 100nM	5.66±0.12	full
Iberiotoxin 100nM	6.44±0.15	full
Apamin 250nM	5.32±0.05	94±15
Glybenclamide 1µM	6.12±0.16	85±4
Capsazepine 1µM	6.28±0.15	95±5
4-AP 3mM	4.93±0.04	91±3
Quinine 200µM	5.32±0.05	full

(*) All pharmacological treatments have been executed on aortic preparations with intact endothelial layer, with the exception of the first and second ones, as indicated in table.

Figure 1

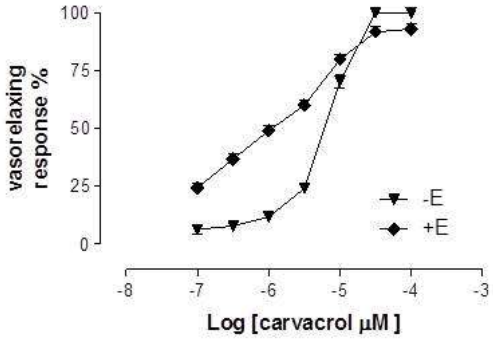
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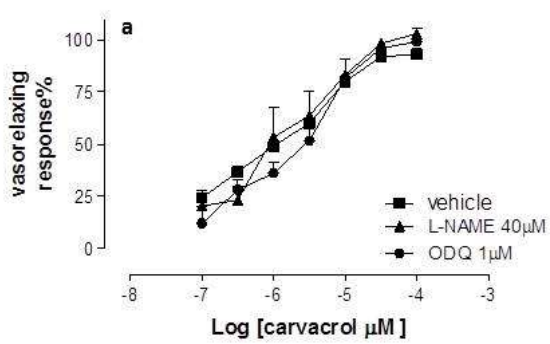
Figure 2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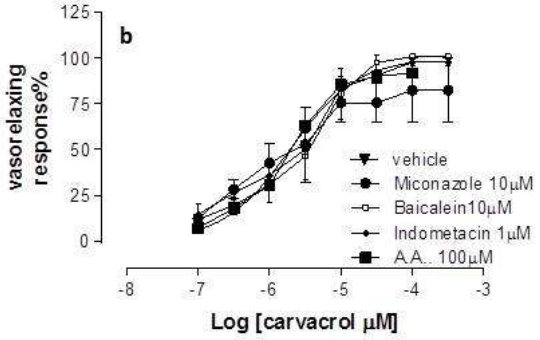
L-NAME 40µM
ODQ 1µM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Indometacin 1µM
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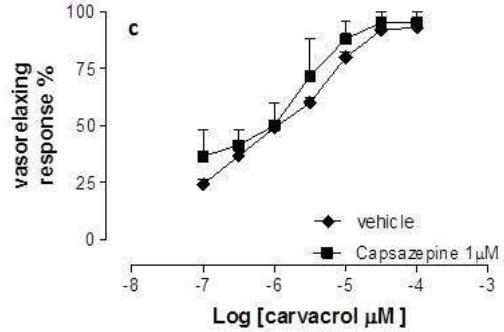
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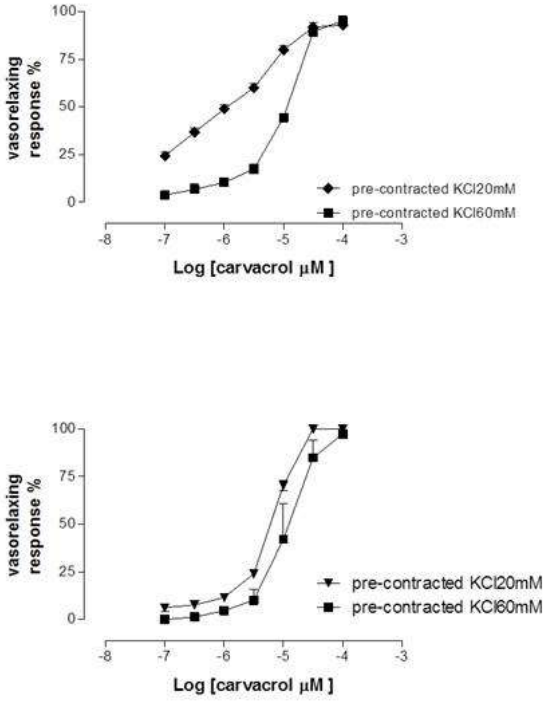
Figure 3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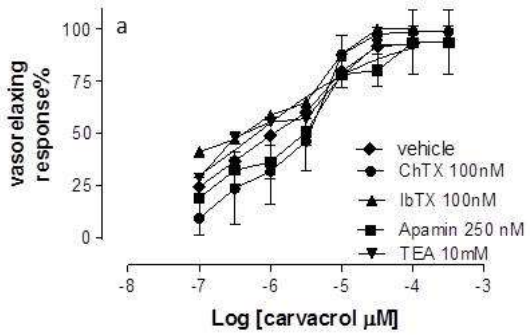
Log [carvacrol μM]
pre-contracted KCl20mM
pre-contracted KCl60mM

Figure 4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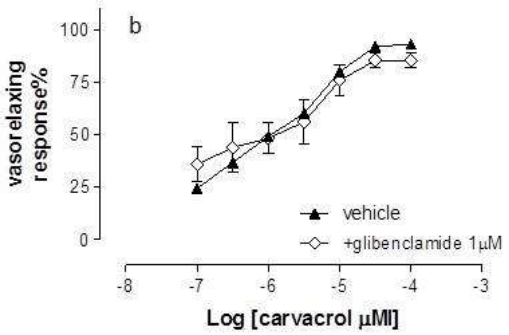
IbTX 100nM
Apamin 250 nM
TEA 10mM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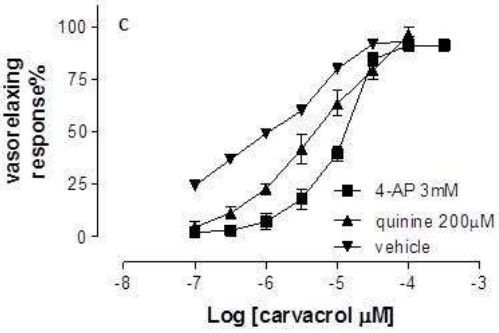
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Log [carvacrol μM]
4-AP 3mM
quinine 200μM
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