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In vitro effect of sanguinarine alkaloid on binding of [^{3}H]candesartan to the human angiotensin AT_1 receptor

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Abstract

The type of interaction of 5-methyl-2,3,7,8-bis(methylenedioxy)benzo[*c*]phenanthridinium (sanguinarine), an alkaloid isolated from the root of *Bocconia frutescens* L., with the human angiotensin AT_1 receptor was evaluated in both intact cells and membrane binding of [^{3}H](2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid) ([^{3}H]candesartan). The results indicate that the inhibition of [^{3}H]candesartan binding by sanguinarine is independent of cell viability, since the alkaloid inhibited at a similar extent radioligand binding on both intact Chinese hamster ovary (CHO) cells transfected with the human angiotensin AT_1 receptor (hAT_1) and their cell membranes ($K_i = 0.14$ and 1.10 μM, respectively). The unsuccessful recovery of [^{3}H]candesartan binding after washing sanguinarine off the cells suggested a nearly irreversible or slow reversible interaction. Saturation binding studies showed a substantial reduction of the B_{max} without affecting the K_d. In addition, the presence of 2-*n*-butyl-4chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan) could not prevent sanguinarine inhibition of [^{3}H]candesartan binding neither. The present findings indicate that sanguinarine interacts with the receptor in a slow, nearly irreversible and noncompetitive manner.

Keywords: Sanguinarine; Angiotensin AT_1 receptor; CHO cell; CHO membrane; Noncompetitive atagonist; [^{3}H]Candesartan

1. Introduction

High blood pressure and pathological mechanisms in vascular diseases might be mediated by angiotensin AT_1 receptors (Matsusaka and Ichikawa, 1997). A previous study of different Panamanian plants used in folk medicine for the treatment of high blood pressure showed that the alcoholic extract of the root of *Bocconia frutescens* L. (Papaveraceae) inhibited the [^{3}H]angiotensin II binding to the angiotensin AT_1 receptor in Chinese hamster ovary (CHO) cells stably transfected with the cDNA for this receptor (Caballero-George et al., 2001). Later on, 5-methyl-2,3,7,8-bis(methylenedioxy)benzo[*c*]phenanthridinium (sanguinarine) was found to be the most active component of the extract with a K_i value of 1.29 μM (Caballero-George et al., 2002).

In the present study, the highly specific and selective angiotensin AT_1 receptor antagonist 2-ethoxy-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid (candesartan) was employed to study the binding properties of sanguinarine for this receptor. In this study, [^{3}H]candesartan was prefered over [^{3}H]angiotensin II in order to avoid the phenomenon of internalization of the receptor due to the binding of angiotensin II.

2. Materials and methods

2.1. Materials

Detailed experimental procedures for the collection of samples, their taxonomic identification, isolation and structure solution of the pure natural product 5-methyl-2,3,7,8-bis(methylenedioxy)benzo[*c*]phenanthridinium (sanguinarine) used in this study are given in an earlier publication

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(Caballero-George et al., 2002). Sanguinarine was dissolved in a minimal amount of dimethyl sulfoxide (DMSO) and water to a concentration 10 × higher than the final concentration in the assay and tested at different concentrations starting from 0.1 mM onwards. The DMSO final concentration did not exceed 1%. 2-Ethoxy-1-[(2′-(1*H*-tetrazol-5-yl) biphenyl-4-yl)methyl]-1*H*-benzimidazoline-7-carboxylic acid (candesartan; Shibouta et al., 1993; Noda et al., 1995), 2-*n*-butyl-4chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole (losartan; Wong et al., 1990) and [^{3}H]candesartan (17 Ci mmol/l) were obtained from Astra Zeneca (Sweden).

2.2. Cells

Wild-type Chinese hamster ovary cells (CHO-K_1) stably transfected with the cDNA for the human angiotensin AT_1 receptor (denoted as CHO-hAT_1 cells) were obtained as described by Vanderheyden et al. (1998). The cells were cultured in 75-cm^2 flasks in Dulbecco's modified essential medium (DMEM) which was supplemented with 2 mM L-glutamine, 2% of a stock solution containing 5000 IU/ml penicillin and 5000 μg/ml streptomycin (Life Technologies, Merelbeke, Belgium) and 10% fetal bovine serum (Life Technologies). The cells were grown in 5% CO_2 at 37 °C until confluent.

2.3. [^{3}H]Candesartan binding

The binding of [^{3}H]candesartan to the hAT_1 receptors on adherent intact CHO-hAT_1 cells in 24-well plates was measured as described previously (Fierens et al., 1999). Before the incubation, each well contained a confluent monolayer of adherent CHO-hAT_1 cells (approximately 0.475 ± 0.05 mg protein/well) and 400 μl DMEM. Competition experiments were carried out by pre-incubation of the cells with 50 μl of the test compounds for 30 min, followed by addition of 50 μl of a final concentration of 1.5 nM [^{3}H]candesartan and a further incubation for 30 min. Alternatively, the test compounds and the radioligand were added simultaneously to the cells (co-incubation). Non-specific binding of the radioligand was measured by co-incubation of the cells with 1 μM (final concentration) unlabeled candesartan. All incubations were carried out at 37 °C.

In saturation binding experiments, CHO-hAT_1 cells were incubated with final concentrations of [^{3}H]candesartan ranging between 0.3 and 10 nM either in the absence (total binding) or in the presence of 1 μM unlabeled candesartan (nonspecific binding). Specific binding of [^{3}H]candesartan was measured in the presence or absence of 10 μM (final concentration) sanguinarine after 30 min incubation.

To assess the rate of the inhibitory effect of sanguinarine, CHO-hAT_1 cells were pre-incubated with a final concentration of 10 μM sanguinarine for the indicated periods of time, after which 1.5 nM (final concentration) [^{3}H]candesartan was added and the cells were further incubated for 30 min.

To evaluate the protective effect of losartan, cells were pre-incubated for 30 min with sanguinarine (3 or 10 μM final concentration), either without or after a 5-min pre-exposure with 10 μM (final concentration) losartan. Subsequently, the cells were washed twice with HEPES buffer (containing HEPES 20 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 2.1 mM, NaCl 137 mM, KCl 2.7 mM, pH 7.4) at 37 °C and then further incubated with 1.5 nM (final concentration) [^{3}H]candesartan for another 30 min.

The incubations were stopped by washing each well three times with 500 μl ice-cold HEPES buffer. The cells were then solubilized with 0.4 M NaOH and transferred to scintillation vials; 3.0 ml of scintillation liquid (Optifase of Wallac, Turku, Finland) was added and the samples were counted for 3 min in a liquid scintillation counter (Wallac, 1219 Rackbeta).

Competition binding assays on CHO-hAT_1 cell membrane preparations were performed as above but starting with 400 μl membranes (≈ 0.5 mg protein/ml) in glass tubes. Membranes were prepared as described previously (Fierens et al., 2001). Briefly, cultured CHO-hAT_1 cells were harvested by trypsinization. The harvested cells were centrifuged (room temperature, 5 min at 500 × *g*), washed twice with cold HEPES buffer and then homogenized by a Polytron homogenizer (3 × 30 s). The resulting homogenate was centrifuged (4 °C, 20 min at 27,000 × *g*) and the supernatant removed. The pellet was resuspended in ice-cold HEPES buffer and the procedure repeated twice. Pre-incubation of the membranes for 30 min with different concentrations of the compounds was followed by incubation for 30 min more with 1.5 nM (final concentration) [^{3}H]candesartan. Adding 3.0 ml ice-cold HEPES buffer stopped the incubation. The membranes were subsequently filtered and washed on Whatman GF/B filters. The filters were placed in vials with 3.0 ml of scintillation liquid and counted for 3 min in a scintillation counter.

2.4. [^{3}H]Thymidine uptake

Cell viability was measured by the active uptake of thymidine as previously described (Fierens et al., 2001). Briefly, confluent cells incubated in 24-well plates overnight with sterile supplement DMEM without serum (stop cell growth) were washed twice with 0.5 ml DMEM buffer and left 15 min with 0.4 ml of supplement DMEM with serum (cells start to grow again). The effect of the alkaloids on [^{3}H]thymidine uptake was measured by pre-incubating the cells for 30 min with different concentrations of the compounds, 10% of ethanol for the nonspecific binding (total cell death) and DMEM medium for the control. Next, cells were incubated with 50 μl of 70 nM (final concentration) [^{3}H]thymidine for 30 min. After the incubation, cells were placed on ice and washed twice with ice-cold HEPES buffer.

Radioactivity was measured like with [^{3}H]angiotensin II binding.

2.5. Data analysis

All results are represented as means ± standard error of the mean (S.E.M.) of at least two independent experiments (each performed in duplicate or triplicate). The calculation of IC_{50} values from competition binding experiments was performed by nonlinear regression analysis using Graph Pad Prism (San Diego, CA, USA). K_i values are calculated from the IC_{50} values according to Cheng and Prusoff (1973) and by using the K_d value of [^{3}H]candesartan as reported previously in the same intact cells (Fierens et al., 1999) and membranes (Fierens et al., 2001).

3. Results

The present results showed the inhibition of [^{3}H]candesartan binding to the human angiotensin AT_1 receptor by sanguinarine. In all binding assays, [^{3}H]candesartan was used as radioligand, which displayed a high affinity for angiotensin AT_1 receptor binding and did not cause internalization of the receptor after binding (Fierens et al., 2001). In this context, it has indeed been shown that nonpeptide antagonists (such as candesartan) do not cause internalization of the angiotensin AT_1 receptor after binding (Fierens et al., 2001). In addition, [^{3}H]angiotensin II and [^{3}H]candesartan gave the same K_i values in competition binding studies (Fierens et al., 1999) and the latest one could be used on both membranes and intact CHO cells (Fierens et al., 2001).

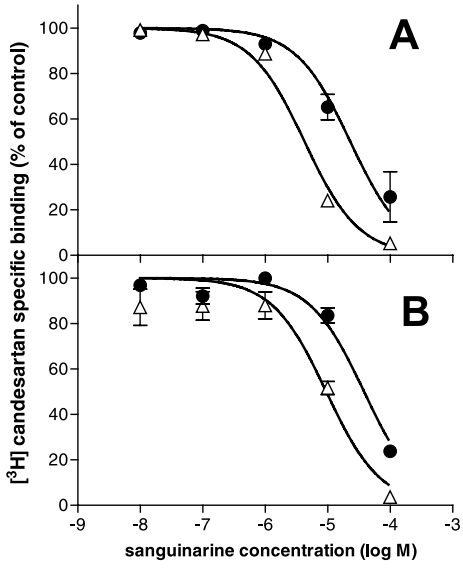

Fig. 1. Inhibition of [^{3}H]candesartan binding to CHO-hAT_1 cells (A) and membranes (B) by different concentrations of sanguinarine. Cells and membranes were either pre-incubated (△) 30 min with the test compound before addition of the radioligand or co-incubated (●) 30 min with both the test compound and the ligand. The corresponding IC_{50} values were 4.37 μM (pre-incubation) and 23.94 μM (co-incubation) in cells (A), and 9.39 μM (pre-incubation) and 38.28 μM (co-incubation) in membranes. The calculated K_i values were 0.14 μM (pre-incubation) and 0.79 μM (co-incubation) in cells (A), and 1.10 μM (pre-incubation) and 4.50 μM (co-incubation) in membranes. Data are the means ± S.E.M. of two independent experiments.

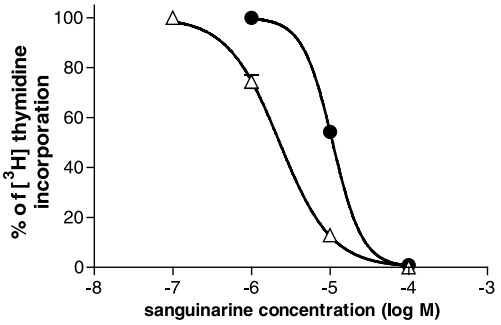

Fig. 2. Determination of [^{3}H]thymidine incorporation on intact CHO-hAT_1 cells after pre-incubation (△) with the test compound before adding the ligand or co-incubation (●) with both the test compound and the ligand. The corresponding IC_{50} values were 2.28 μM (pre-incubation) and 10.78 μM (co-incubation). Data are presented as the means ± S.E.M. of two independent experiments.

Fig. 1 shows the inhibition of [^{3}H]candesartan binding by different concentrations of sanguinarine. The IC_{50} value after pre-incubation with this compound was 4.37 μM (K_i = 0.14 μM) on intact cells, in the same range as that found in an early investigation on [^{3}H]angiotensin II binding inhibition (Caballero-George et al., 2002), suggesting that sanguinarine somehow interfered with the specific binding of selective ligands to the angiotensin AT_1 receptor.

Since there have been several publications on the potential cytotoxicity of sanguinarine (Babich et al., 1996; Slaninova et al., 2001), the possibility of cell death and subsequent receptor degradation were considered in this study. A viability test was carried out measuring [^{3}H]thymidine incorporation after exposure of the cell monolayer to sanguinarine. Sanguinarine reduced incorporation of labeled thymidine in a dose-dependent manner (Fig. 2), implying a possible cell death at high concentrations.

In a previous study, hypertonic sucrose (0.4 M) affected the binding of candesartan to intact CHO-hAT_1 cells and this effect was at least partially related to its cytotoxicity. Interestingly, sucrose did not cause any effect on membrane binding with candesartan (Fierens et al., 2001). Therefore, the inhibition of [^{3}H]candesartan binding by sanguinarine was measured in both intact cells (Fig. 1A) and membranes (Fig. 1B). The observed effect suggested that sanguinarine indeed interacted with the receptor and that this interaction is likely not to be related with the possible cytotoxicity.

The [^{3}H]candesartan inhibition experiments with sanguinarine were carried out under pre- and co-incubation conditions. For this purpose, the membranes and intact adherent cells were either pre-incubated with sanguinarine before the incubation with [^{3}H]candesartan or both were added simultaneously in the incubation mixture. This was to find out

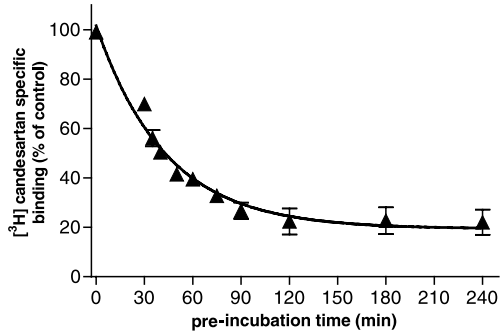

Fig. 3. The time dependency of the sanguinarine (▲) effect (10 μM) on [^{3}H]candesartan (1.5 nM) in CHO-hAT$_1$ cells. Data are the mean values ± S.E.M. of two independent experiments represented as % of the control [^{3}H]candesartan binding in the absence of sanguinarine.

whether the inhibition by sanguinarine is rapidly or slowly reversible or even irreversible. In a previous study, a similar approach was carried out in which the inhibition of angiotensin II-induced angiotensin AT$_1$ receptor activation by nonpeptide antagonists was measured (Fierens et al., 2001). It appeared that upon increasing the pre-incubation time of the antagonists, the resulting curves were either shifted to the left with respect to the curve after co-incubation (in the case of a slowly reversible antagonist, candesartan) or remained unchanged (in the case of the rapidly reversible antagonist, losartan). In both intact adherent cells as well as in membranes (Fig. 1A,B), the [^{3}H]candesartan inhibition curves displayed a leftward shift when sanguinarine was pre-incubated as compared to the simultaneous incubation and therefore indicated that its inhibition was either slowly reversible or even irreversible within the time range of the experiments.

In the same line, washing off the cells could not reverse the inhibitory effect of sanguinarine. In this assay, intact cells were pre-incubated with 10 μM sanguinarine for the indicated periods of time (up to 4 h), after which the cells were washed and subsequently incubated with [^{3}H]candesartan (Fig. 3). Together with the inhibition curves, these

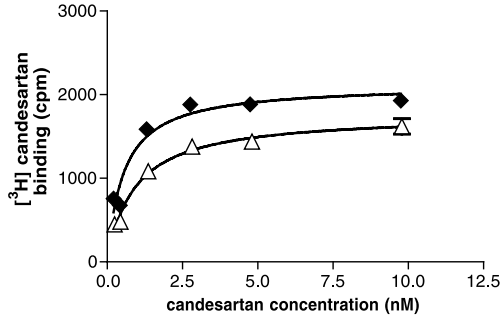

Fig. 4. Effect of sanguinarine on [^{3}H]candesartan saturation binding data to intact CHO-hAT$_1$. Specific binding of [^{3}H]candesartan was measured in the presence (△) or absence (◆) of 10 μM sanguinarine after 30 min incubation at 37 °C. B_{max} values of [^{3}H]candesartan binding were 2120 and 1765 cpm, while K_d were 0.545 and 0.912 nM in the absence and presence of sanguinarine, respectively.

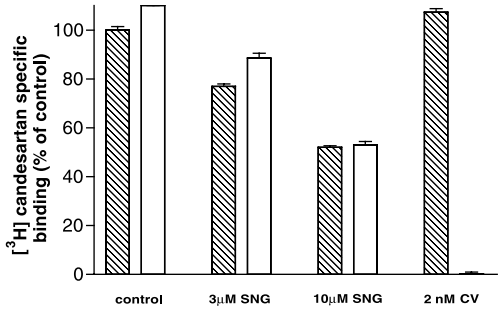

Fig. 5. Effect of losartan in the inhibition of [^{3}H]candesartan binding by sanguinarine. Intact CHO-hAT$_1$ cells were pre-incubated 5 min with 10 μM losartan (▨) or buffer (□) followed by 30 min with 3 or 10 μM sanguinarine. Cells were washed twice and 50 μl DMEM containing 1.5 nM [^{3}H]candesartan for total binding and 2 nM nonlabeled candesartan for nonspecific binding. Values are the means ± S.E.M. of two independent experiments, performed in triplicate each. Control: total [^{3}H]candesartan binding; SNG: sanguinarine.

data suggested the slow reversible or nearly irreversible inhibitory effect of sanguinarine.

Interestingly, sanguinarine produced a substantial reduction of the maximal binding capacity (B_{max}) of [^{3}H]candesartan, without affecting the K_d (Fig. 4). Such an effect is generally interpreted as the result of a noncompetitive (or allosteric) type of inhibition, but this pattern is also observed for competitive but slowly reversible ligands. To discriminate between both competitive and noncompetitive inhibition, the ability of sanguinarine inhibition of [^{3}H]candesartan binding was studied in the absence or presence of the fast reversible and competitive angiotensin AT$_1$ receptor antagonist losartan. The same inhibition of [^{3}H]candesartan binding was seen when cells were pre-incubated with losartan and sanguinarine as compared to pre-incubation with sanguinarine alone (Fig. 5). As the inhibitory effect of sanguinarine could not be prevented by inclusion of 10 μM losartan, these data suggested that sanguinarine may bind to a distinct binding site of the angiotensin AT$_1$ receptor (with respect to the nonpeptide antagonists).

4. Discussion

Our major findings showed that sanguinarine inhibited the binding of specific radioligands to the angiotensin AT$_1$ receptor in the micromolar range. Although sanguinarine inhibited cell viability (measured by the uptake of [^{3}H]thymidine in intact cells) at a similar concentration range as the inhibition of [^{3}H]candesartan binding, our results exhibited that [^{3}H]candesartan binding was not restricted to intact cells, but also found on membranes indicating that there is no strict requirement to have apoptosis or cytotoxicity to perceive the inhibition of angiotensin AT$_1$ receptor binding. In the same line, a recent study shows that sanguinarine causes apoptosis, but only in particular tumor cells and not in other 'normal' cells (Ding et al., 2002). Moreover, this apoptotic effect was only observed after 4 h incubation.

The kinetic experiments shown in Fig. 3 exhibited a slow onset of the inhibition of [^{3}H]candesartan binding by sanguinarine. The reason for this is not clear at the moment, but merits further investigation. The inhibition caused by sanguinarine was not reversible within the time frame used in this experiment. The behavior of sanguinarine beyond 4 h incubation could not be studied with this technique, because these cells cannot survive incubation for longer time. The saturation binding and protection experiments confirmed the slow and/or irreversible binding of sanguinarine, which was noncompetitive.

When compared to other in vitro studies of inhibition of specific ligands by sanguinarine, the same range of activity was found, e.g. the vasopressin V_1 receptor (7 μM) (Granger et al., 1992), adrenoceptors α_1 and α_2 (33.6 and 6.4 μM), 5-HT_2 (91.7 μM), nicotinic (11.8 μM) and muscarinic (2.4 μM) receptors (Schmeller et al., 1997), which are all (except for the nicotinic receptor) G-protein-coupled receptors like the angiotensin AT_1 receptor. Interestingly, these receptors have also been described to play a role in blood pressure regulation (Calabrese, 2001; Hong et al., 1994; Cooper, 2001; Walch et al., 2001). The interaction of sanguinarine with the vasopressin V_1 receptor was claimed to be of competitive nature, while for the other receptors is still unknown.

Sanguinarine has also been found to interact with other subcellular systems in the micromolar range, e.g. enzymes such as acetylcholine (Ulrichova et al., 1983; Schmeller et al., 1997) and butyrylcholine esterase, choline acetyltransferase (Schmeller et al., 1997), Na^+,K^+-ATPase (Scheiner-Bobis, 2000), monoamine oxidase activity (Lee et al., 2001), AMP-dependent protein kinase (Wang et al., 1997), and it has shown inhibiting effects over microtubule function (Wolff and Knipling, 1993) and the nuclear factor-κB (NF-κB) activation (Chaturvedi et al., 1997).

All the studies described above might question the specificity of this compound. Nevertheless, the same extract containing sanguinarine that actively inhibited [^{3}H]angiotensin II binding to the angiotensin AT_1 receptor did not affect [^{3}H]neuropeptide Y binding to the Y_1 receptor (Caballero-George et al., 2001). So the nature of the specificity of sanguinarine is still not completely understood.

The best known actions of sanguinarine are the antimicrobial (Giuliana et al., 1999; Ishikawa, 2001), fungicidal (Giuliana et al., 1997) and the inhibition of dental plaque (Tenenbaum et al., 1999). Nevertheless, sanguinarine possesses other interesting actions such as endothelium-independent induction of vasorelaxation in rat thoracic aorta, which might involve the inhibition of inositol-1,4,5-triphosphate and blockade of calcium channel (Hu et al., 2001). Regardless of the controversial toxic effects of this alkaloid, all these actions are of major importance in studying the targets modulated by sanguinarine and clarify its mechanism of action at the different levels.

This work also emphasized the importance of radioligand binding techniques in the development of in vitro pharmacological studies directed to evaluate plant products.

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