

Exploring the binding-site crevice of a family B GPCR, the type 1 corticotropin releasing  
factor receptor <sup>#</sup>

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### **Abbreviations:**

CRF; corticotropin releasing factor; CRF<sub>1</sub>, type 1 receptor for the corticotropin releasing factor; GPCRs, G-protein-coupled receptors; TM, membrane-spanning segment; SCAM, substituted-cysteine accessibility method; MTS, methanethiosulfonate; MTSEA+, ethylammonium MTS; MTSET+, trimethylammonium MTS; MTSES-, ethylsulfonate MTS; WT wild type CRF<sub>1</sub>; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

## **ABSTRACT**

Family B of G-protein-coupled receptors (GPCRs) is comprised of receptors that bind peptides, such as secretin, glucagon, parathyroid hormone, and corticotropin releasing factor (CRF), which play critical physiological roles. These receptors, like all GPCRs, share a common structural motif of seven membrane-spanning segments, which have been proposed to bind small ligands, such as antalarmin, a non-peptide antagonist of the type 1 receptor for CRF (CRF<sub>1</sub>). This leads to the hypothesis that as for family A GPCRs, the binding sites of small ligands for family B GPCRs are on the surface of a water-accessible crevice, the binding-site crevice, which is formed by the membrane-spanning segments and extends from the extracellular surface of the receptor into the plane of the membrane. To test this hypothesis we have begun to obtain structural information about family B GPCRs, using as prototype the CRF<sub>1</sub>, by determining the ability of sulfhydryl-specific methanethiosulfonate derivatives, such as the MTSethylammonium (MTSEA), to react with CRF<sub>1</sub> and thus irreversibly inhibit [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding. We found that MTSEA inhibited [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding to CRF<sub>1</sub>, and that antalarmin protected against this irreversible inhibition. To identify the susceptible cysteine(s), we mutated, one at a time, four endogenous cysteines to serine. Mutation to serine of Cys211, Cys233, or Cys364 decreased the susceptibility of sauvagine binding to irreversible inhibition by MTSEA. Thus, Cys211, Cys233 and Cys364, at the cytoplasmic ends of the third, fourth and seventh membrane-spanning segments, are exposed in the binding-site crevice of CRF<sub>1</sub>.

## **INTRODUCTION**

Family B of G-protein-coupled receptors (GPCRs) is comprised of receptors that bind functionally important peptides, including corticotropin releasing factor (CRF), a 41-amino acid peptide that plays a major physiological role by regulating the activity of the hypothalamic-pituitary-adrenal axis (Chrousos, 1995; Harmar, 2001; Vale et al., 1981).

Sequence analysis of these receptors has revealed seven putative, mostly hydrophobic, plasma membrane-spanning segments connected by alternating intracellular and extracellular loops (Gether, 2000; Grigoriadis et al., 2001; Harmar, 2001). These receptors also have a large extracellular amino-terminal region (N-region) that has been structurally characterized in recent NMR and crystallographic studies (Grace et al., 2007; Pioszak et al., 2008; Underwood et al., 2010). The N-region and the extracellular loops of family B GPCRs have been shown to play an important role in peptide binding (Assil-Kishawi et al., 2008; Dautzenberg et al., 1999; Gkountelias et al., 2009; Grace et al., 2007; Holtmann et al., 1996; Kraetke et al., 2005; Liaw et al., 1997; Perrin et al., 1998; Pioszak et al., 2008; Unson et al., 2002).

In contrast to the extracellular regions, little is known about the potential role in ligand binding of the membrane-spanning segments (TMs) of family B GPCRs. Although the TMs have been proposed to bind small non-peptide ligands, such as antalarmin, an antagonist for the type 1 CRF receptor (CRF<sub>1</sub>), the specific interactions have not been identified (Hoare et al., 2003; Liaw et al., 1997). In contrast to the TMs of family A GPCRs, which have been structurally characterized in multiple crystallographic, biophysical and biochemical studies, there is no structural information about the TMs



of family B GPCRs, further complicating a determination of their role in ligand binding. Importantly, family B GPCRs display very little sequence similarity with family A receptors, and their TMs do not share the common structural/functional motifs identified in the latter (Donnelly, 1997; Frimurer and Bywater, 1999; Gether, 2000). All these factors hinder the construction of accurate molecular models of family B GPCRs. Nevertheless, based on sequence analysis several models of these receptors have been created (Donnelly, 1997; Frimurer and Bywater, 1999). Despite the assumptions required and the associated uncertainties of the exact boundaries of the TMs and their orientations, the overall TM packing of family B GPCRs has been proposed to be similar to that of family A GPCRs. This, in conjunction with their role in the binding of small non-peptide ligands led us to hypothesize that as in the family A GPCRs, the TMs of family B receptors form a water-filled binding-site crevice, which extends from the extracellular surface of the receptor into the plane of the membrane (Javitch et al., 1994). The surface of this crevice is formed by residues that contact ligands, as well as, by other residues that may play a structural role and affect binding indirectly.

To test this hypothesis we sought to obtain structural information for the TMs of family B GPCRs, using as a prototype the CRF<sub>1</sub>. Our starting point was to test whether one or more of the endogenous TM cysteines in CRF<sub>1</sub> face the putative binding-site crevice by determining their accessibilities for reaction with small, charged, sulfhydryl-specific methanethiosulfonate (MTS) derivatives. These reagents react vastly faster with water-accessible sulfhydryl groups of cysteines than with sulfhydryls buried in the protein or facing the lipid bilayer (Karlin and Akabas, 1998). Using this method previous studies have revealed the presence of endogenous TM cysteines that face the binding-site

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crevice of various family A GPCRs (Deng et al., 2000; Javitch et al., 1994). Here we found that the endogenous Cys211, Cys233 and Cys364 in the third, fourth and seventh membrane-spanning segments of CRF<sub>1</sub> are located on the surface of a binding-site crevice of CRF<sub>1</sub>, being accessible to reaction with charged polar MTS reagents and protected from this reaction by bound antalarmin.

## MATERIALS AND METHODS

**Plasmids and site-directed mutagenesis.** The cDNA sequence encoding the type 1 human corticotropin releasing factor receptor (CRF<sub>1</sub>) was subcloned into the bicistronic expression vector pcin4, thereby creating the vector pcin4-CRF<sub>1</sub> (Gkountelias et al., 2009). Serine mutations were generated by the polymerase chain reaction (PCR)-mediated mutagenesis, using Pfu polymerase (Fermentas USA) and mutagenic oligonucleotides encoding the desired amino acid substitution. The PCR-generated DNA fragments containing the mutations were subcloned into the pcin4-CRF<sub>1</sub> plasmid and the mutations were confirmed by DNA sequencing. Mutants are named as (wild-type residue)(residue number)(serine), where the residues are given in the single-letter code.

**Cell culture, transfection and harvesting.** Human embryonic kidney cells (HEK 293) were grown in DMEM/F12 (1:1) containing 3.15 g/L glucose and 10% bovine calf serum at 37 °C and 5% CO<sub>2</sub>. Sixty mm dishes of HEK 293 cells at 80-90% confluence were transfected with 2-3 mg of wild type (WT) or mutant pcin4-CRF<sub>1</sub> using 9 µl of Lipofectamine (Invitrogen, USA) and 2 ml of OPTIMEM (Invitrogen, USA). To generate stably transfected pools of cells expressing the receptors five to twelve hours after transfection, the medium was replaced by DMEM/F12 (1:1) containing 3.15 g/L glucose, 10% bovine calf serum (Hyclone, batch #APD21173) and 700 µg/ml of the antibiotic, Geneticin (Invitrogen, USA). The antibiotic was added to select a stably transfected pool of cells. Cells stably expressing WT or CRF<sub>1</sub> mutants, at 100% confluence in 60 mm or 100 mm dishes, were washed with phosphate-buffered saline (PBS) (4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, pH 7.3-7.4 at 37°C),

briefly treated with PBS containing 2 mM EDTA (PBS/EDTA), and then dissociated in PBS/EDTA. Cells suspensions were centrifuged at 50 x g for 2 min at room temperature, and the pellets were resuspended in 1 ml of buffer M (25 mM HEPES, containing 5.4 mM KCl, 140 mM NaCl, 2 mM EDTA, pH 7.2 at 22-25°C) for treatment with MTS reagents or in 1.5 ml of buffer H (20 mM HEPES, containing 10 mM MgCl<sub>2</sub>, 2 mM EGTA, 0.2 mg/ml bacitracin and 0.93 µg/ml aprotinin pH 7.2 at 4°C) for binding assays.

**[<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding.** For radioligand binding assays cell suspensions (1.5 ml) in buffer H were homogenized using a Janke & Kunkel IKA Ultra Turrax T25 homogenizer, at setting ~20, for 10-15 s, at 4 °C. The homogenates were centrifuged at 16000 x g, for 10 min, at 4 °C and the membrane pellets were resuspended in 1 ml buffer B (buffer H containing 0.1% BSA, pH 7.2 at 20°C). The membrane suspensions were diluted in buffer B and used for homologous competition binding studies as previously described (Gkountelias et al., 2009). Briefly, aliquots of diluted membrane suspensions (50 µl) were added into low retention tubes (Kisker, Germany), containing buffer B and 20-25 pM [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine with or without increasing concentrations of Tyr<sup>0</sup>-sauvagine (American Peptide Co. Inc, USA). The mixtures were incubated at 20-21 °C for 120 min and then filtered using a Brandel cell harvester through Whatman 934AH glass fiber filters, presoaked for 1 hr in 0.3% polyethylenimine at 4 °C. The filters were washed 3 times with 0.5 ml of ice-cold PBS, pH 7.1 containing 0.01% Triton X-100. Filters were assessed for radioactivity in a gamma counter (LKB Wallac 1275 minigamma, 80% efficiency). The amount of membrane used was adjusted to insure that the specific binding was always equal to or less than 10% of the total concentration of the

added radioligand. Specific [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine binding was defined as total binding less nonspecific binding in the presence of 500-1000 nM CRF. The K<sub>D</sub> values for  $^{125}$ I-Tyr<sup>0</sup>-sauvagine binding were determined by analyzing homologous competition data with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

**Reactions with MTS Reagents.** For treatment with MTS reagents, aliquots (0.1 ml) of cell suspensions in buffer M were incubated with the methanethiosulfonate (MTS) reagents, MTSethylammonium (MTSEA), MTSethyltrimethylammonium (MTSET), or MTSethysulfonate (MTSES), at the stated concentrations for 15 sec at 22-25 °C. Cell suspensions were then diluted 140-fold in buffer PBS/EDTA (pH 7.1 at 22-25°C), containing 10 mM MgCl<sub>2</sub>, centrifuged at 250 x g, for 5 min, at 22-25 °C and the pellets were resuspended in 1.5 ml of buffer M, containing 10 mM MgCl<sub>2</sub>. Cell suspensions were centrifuged at 250 x g, for 5 min, at 22-25 °C and the pellets were homogenized in 1.5 ml of buffer H, as described above. The homogenates were centrifuged at 16000 x g, for 10 min, at 4 °C and the membrane pellets were resuspended in 1ml buffer B (buffer H containing 0.1% BSA, pH 7.2 at 20°C). The membrane suspensions were used to assay for [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine binding as described above.

Protection experiments were performed by preincubation of aliquots (0.1 ml) of cell suspensions with increasing concentrations of the non-peptide CRF<sub>1</sub>-selective antagonist (1-1000 nM), antalarmin, for 30 min at 37°C in a final volume of 1 ml buffer M. Subsequently the mixtures were centrifuged at 250 x g for 5 min at 22-25°C, and 0.9 ml of supernatant was removed by aspiration. The cell pellets were resuspended in the remaining 0.1 ml of supernatants and the mixtures were treated with 2.5 mM MTSEA as

described above. Cells were subsequently diluted 140-fold in buffer PBS/EDTA (pH 7.1 at 22-25°C), containing 10 mM MgCl<sub>2</sub>, washed twice by centrifugation with buffer M containing 10 mM MgCl<sub>2</sub>, and membrane homogenates were prepared and used to assay for [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding as described above.

## RESULTS

**Reaction of MTS-reagents with the CRF<sub>1</sub>.** To assess for the reaction of MTS reagents with the CRF<sub>1</sub> we treated intact HEK 293 cells stably expressing wild type (WT) CRF<sub>1</sub> with MTSEA, MTSET or MTSES and subsequently determined the specific binding of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine in membrane homogenates.

Treatment of HEK 293 cells stably expressing CRF<sub>1</sub> with the positively charged MTSEA at a concentration of 2.5 mM significantly decreased the specific binding of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine to CRF<sub>1</sub> (Fig. 1). In contrast to MTSEA, the bulkier MTSET (positively charged) and MTSES (negatively charged), at concentrations of 2.5 mM, did not significantly reduce the specific binding of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine to CRF<sub>1</sub> (Fig. 1). As shown in Fig.2, MTSEA treatment decreased the specific [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding in a dose-dependent manner with an IC<sub>50</sub> of 2.1 mM (-logIC<sub>50</sub> = 2.69 ± 0.18), reaching a plateau of about 20% residual specific binding. Longer treatment of CRF<sub>1</sub> with 10 mM MTSEA for 2 min demonstrated a similar plateau in radioligand binding (data not shown).

**Mechanism of the inhibitory effect of MTSEA on binding.** To examine the mechanism of the inhibitory effect of MTSEA on [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding we determined the binding affinity of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine before and after the reaction of MTSEA with CRF<sub>1</sub>. As shown in Fig. 3 treatment of WT CRF<sub>1</sub> with either 2.5 mM or 15 mM MTSEA failed to significantly affect [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding affinity. Thus inhibition of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding after reaction of CRF<sub>1</sub> with MTSEA resulted from a reduction in the apparent number of functional binding sites, consistent with loss of

binding to receptor that was fully derivatized. A possible explanation for the small fraction of residual binding with normal affinity despite treatment with saturating MTSEA could be that a small proportion of receptors was located intracellularly and thus protected from the reagent, whereas the binding experiments were performed with membrane homogenates in which [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine labeled all receptors.

**Protection of CRF<sub>1</sub> against MTSEA reaction.** To test whether the small non-peptide CRF<sub>1</sub>-selective antagonist, antalarmin, protected the WT CRF<sub>1</sub> against MTSEA reaction, we determined its ability to slow the reaction. We treated CRF<sub>1</sub>-expressing cells, incubated with or without antalarmin, with MTSEA (2.5 mM), and, after washing, determined [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine binding in membrane homogenates from these cells. As shown in Fig. 4, antalarmin protected CRF<sub>1</sub> against MTSEA reaction in a concentration-dependent manner, with complete protection at a high concentration of the ligand.

**Mutations of the endogenous Cys of CRF<sub>1</sub>.** To identify the endogenous Cys of CRF<sub>1</sub> that reacted with MTSEA to inhibit binding, we mutated to Ser, one at a time, the endogenous Cys128, Cys211, Cys233 and Cys364 (thus creating the C128S, C211S, C233S and C364S mutants). According to the predicted topology of CRF<sub>1</sub>, these Cys are located in the first (TM1), third (TM3), fourth (TM4) and seventh (TM7) membrane-spanning segments of receptor (Fig. 5) (Grigoriadis et al., 2001).

Before probing the reaction of MTSEA with the C128S, C211S, C233S and C364S constructs, we tested the impact of the mutations on the functional properties of CRF<sub>1</sub>, by determining the binding affinities ( $-\log K_D$ ) of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine for WT and



the mutant receptors in homologous competition experiments performed under equilibrium conditions in membrane homogenates from HEK 293 cells stably expressing the receptors. Substitution of Ser for Cys128, Cys211, Cys233 and Cys364 did not significantly affect the binding affinity of  $^{125}\text{I}$ -Tyr<sup>0</sup>-sauvagine for CRF<sub>1</sub> (Fig. 6), suggesting that the mutations did not substantially alter the functional properties of the receptor.

**Reaction of MTSEA with CRF<sub>1</sub> mutants.** To identify the reactive Cys of CRF<sub>1</sub>, we determined whether the binding of [ $^{125}\text{I}$ ]-Tyr<sup>0</sup>-sauvagine to C128S, C211S, C233S and C364S mutants was sensitive to MTSEA. As shown in Fig.7, mutation of Cys128 to Ser did not affect the sensitivity of radioligand binding to MTSEA; the residual [ $^{125}\text{I}$ ]-Tyr<sup>0</sup>-sauvagine binding to C128S ( $52.5 \pm 3.3\%$ ) after MTSEA treatment (2.5 mM) was not significantly different than that to WT CRF<sub>1</sub> ( $57.4 \pm 2.9\%$ ). In marked contrast, mutation to Ser of Cys211, Cys233 or Cys364 significantly reduced sensitivity to MTSEA; the residual binding of [ $^{125}\text{I}$ ]-Tyr<sup>0</sup>-sauvagine to C211S ( $73.3 \pm 4.5\%$ ), C233S ( $75.9 \pm 9.9\%$ ), and C364S ( $83.0 \pm 9.4\%$ ) after MTSEA treatment (2.5 mM) was not significantly different, but each was significantly different from that of WT ( $57.4 \pm 2.9\%$ ) (Fig 7).

Since radiolabelled sauvagine has been shown to bind to the extracellular portion of CRF<sub>1</sub> and thus is located at some distance from the endogenous cysteines being derivatized, the effects on binding must be indirect. To explore the mechanism of inhibition, we determined the affinity of [ $^{125}\text{I}$ ]-Tyr<sup>0</sup>-sauvagine binding to C128S, C211S, C233S and C364S (and to the other mutants tested in this study, as described below) before and after MTSEA reaction. As was the case for WT, MTSEA reaction did not

significantly affect the binding affinity of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine for C128S, C211S, C233S or C364S (Fig. 6). Thus, as for WT, the decrease of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine binding to C128S after MTSEA reaction was due to a reduction of the apparent number of binding sites. In addition, the much smaller impact of MTSEA reaction with C211S, C233S or C364S on [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine binding was not due to a MTSEA-associated enhancement of the affinity of residual radioligand binding.

**MTSEA reaction with  $\Delta$ Cys mutants.** Based on the finding that substitution of Ser for a single Cys at positions 211, 233 or 364 rendered the receptor much less sensitive to MTSEA, we hypothesized that reaction with MTSEA of only a single one of these Cys, would not reduce sauvagine binding to CRF<sub>1</sub>. To test this hypothesis, and even more importantly to create a suitable MTSEA-insensitive background construct for subsequent substituted-cysteine accessibility method (SCAM) studies, we mutated all the endogenous TM Cys (along with the cytoplasmic Cys150) to Ser, thereby creating the mutant  $\Delta$ Cys, which had a binding affinity for [ $^{125}$ I]-sauvagine ( $-\log K_D = 8.60 \pm 0.63$ ), similar to that of WT ( $-\log K_D = 9.05 \pm 0.08$ ), (Fig. 8). As anticipated, simultaneous mutation of all the sensitive Cys in CRF<sub>1</sub> to Ser created a receptor less sensitive to 2.5 mM MTSEA; the residual binding of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine to  $\Delta$ Cys after MTSEA (2.5 mM) reaction was  $90.9 \pm 6.1\%$  (Fig 9). Consistent with our prediction, we found that addition of a single Cys into  $\Delta$ Cys (at positions, 128, 211, 233 or 364, thus creating the mutants  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C and  $\Delta$ Cys + 364C, respectively) failed to increase significantly the sensitivity to MTSEA. Thus, the residual binding of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine to  $\Delta$ Cys + 128C ( $83.1 \pm 7.6\%$ ),  $\Delta$ Cys + 211C ( $86.0 \pm 9.1\%$ ),  $\Delta$ Cys

+ 233C ( $89.4 \pm 7.7\%$ ) and  $\Delta$ Cys + 364C ( $76.7 \pm 7.3\%$ ) after MTSEA (2.5 mM) reaction was not significantly different from each other or from that of  $\Delta$ Cys ( $90.9 \pm 6.1\%$ ), but it was significantly different from the corresponding binding to WT ( $57.4 \pm 2.9\%$ ) (Fig 9). These observations were not due to a mutation-associated or to an MTSEA-induced change of [ $^{125}$ I]-Tyr<sup>0</sup>sauvagine affinity, since the  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C and  $\Delta$ Cys + 364C mutants, similar to  $\Delta$ Cys, had normal [ $^{125}$ I]-Tyr<sup>0</sup>sauvagine affinity, which also was unaltered by MTSEA treatment (Fig 8).

We next hypothesized that derivatization of all three endogenous cysteines (Cys211, Cys233 and Cys364) is necessary for the reduction of sauvagine binding to CRF<sub>1</sub> after MTSEA reaction. To test this hypothesis we simultaneously added two or three cysteines (at positions 211, 233 and/or 364) into  $\Delta$ Cys and tested their ability to react with MTSEA. Simultaneous addition of two Cys into  $\Delta$ Cys, in any combination, thus creating the mutants  $\Delta$ Cys+233C+364C,  $\Delta$ Cys+211C+364C and  $\Delta$ Cys+211C+233C, did not significantly increase the sensitivity to MTSEA; although the residual binding of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine to  $\Delta$ Cys+233C+364C, ( $93.6 \pm 13.0\%$ ),  $\Delta$ Cys+211C+364C ( $101.3 \pm 9.9\%$ ) and  $\Delta$ Cys+211C+233C ( $96.8 \pm 7.8\%$ ) after MTSEA (2.5 mM) reaction was not significantly different from each other or from  $\Delta$ Cys ( $90.9 \pm 6.1\%$ ), it was significantly different from the corresponding binding to WT ( $57.4 \pm 2.9\%$ ) (Fig.11). In marked contrast, simultaneous addition of the three Cys into the  $\Delta$ Cys, thus creating the mutant,  $\Delta$ Cys+211C+233C+364C, synergistically increased the sensitivity of the mutant receptor to MTSEA, restoring the wild type phenotype. The residual binding of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine to  $\Delta$ Cys+211C+233C+364C ( $61.6 \pm 5.1\%$ ), after MTSEA treatment (2.5 mM) was not significantly different from the corresponding binding to WT ( $57.4 \pm 2.9\%$ ), but

it differed significantly from that to  $\Delta$ Cys ( $90.9 \pm 6.1\%$ ) (Fig.11). These findings were not due to a mutation-associated or to an MTSEA-induced change of [ $^{125}$ I]-Tyr<sup>0</sup>sauvagine affinity, since the mutants had normal [ $^{125}$ I]-Tyr<sup>0</sup>sauvagine affinity, which also was unaltered by MTSEA treatment (Fig. 10). These results also suggest that, similar to WT, the observed significant decrease of [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine binding to  $\Delta$ Cys+211C+233C+364C after MTSEA reaction was due to a reduction of the apparent number of binding sites rather than to a decrease of its affinity.

## **DISCUSSION**

Reaction of MTSEA with CRF<sub>1</sub> decreased the specific binding of the radiolabelled agonist, [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine, suggesting that one or more endogenous Cys was accessible for reaction with the reagent. CRF<sub>1</sub> contains thirteen endogenous Cys (Fig.5). Four of these (Cys128, Cys211, Cys233, Cys364) are predicted to be in the TMs of CRF<sub>1</sub> (TM Cys), one is intracellular (Cys150), whereas six (Cys30, Cys44, Cys54, Cys68, Cys87 and Cys102) are located in the extracellular N-region of CRF<sub>1</sub> and form three disulfide bonds (Pioszak et al., 2008). In addition, two Cys (Cys188, and Cys258) in the first and second extracellular loops, which are highly conserved among the G-protein coupled receptors, likely participate in the formation of a disulfide bond (Qi et al., 1997). Since the MTS reagents do not react with disulfide-bonded Cys, and have limited access to intracellular Cys, if added extracellularly to intact cells with high intracellular reducing environment and for a short period of time (15 sec in our study) (Javitch et al., 1994; Javitch et al., 2002), the endogenous Cys of CRF<sub>1</sub>, which reacted with MTSEA and inhibited [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding are most likely one or more of the four TM Cys (Cys128 in the middle of TM1, and Cys211, Cys233, Cys364, which are located near the cytoplasmic ends of TM3, TM4 and TM7, respectively. This suggests that as in family A GPCRs, the TMs of family B GPCRs, form a water-accessible crevice, with one or more of the endogenous Cys128, Cys211, Cys233 and Cys364 of CRF<sub>1</sub> lying on its surface.

In contrast to MTSEA, MTSET and MTSES did not significantly inhibit [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding to CRF<sub>1</sub>. A possible explanation could be that the reactive endogenous Cys are deep in the crevice such that access of the bulkier MTSET and MTSES is sterically constrained. Similarly, although the cysteines substituted for Ser129

in the cytoplasmic end of TM3 and for Val378 in the cytoplasmic end of TM6 of D2 dopamine receptor were accessible for reaction with MTSEA, they did not react with the bulkier MTSET and MTSES (Javitch et al., 1998; Javitch et al., 1995). An alternative explanation for the ability of MTSEA but not MTSET or MTSES to react is that MTSEA might access the site in its uncharged form, which cannot occur for MTSET or MTSES.

To identify the endogenous Cys that reacted with MTSEA we mutated Cys128, Cys211, Cys233 and Cys364, one at a time, to Ser, thus creating the mutants, C128S, C211S, C233S and C364S. These mutations did not appear to alter significantly the functional and therefore the structural properties of CRF<sub>1</sub>. In contrast to C128S, the mutations, C211S, C233S and C364S rendered the receptor substantially less sensitive to MTSEA. These results suggest that Cys211 in TM3, Cys233 in TM4 and Cys364 in TM7 are exposed in the water-accessible crevice of receptor and reacted with MTSEA to inhibit [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine binding. It is also conceivable that these amino-acids are located in a water-accessible interface formed by the membrane spanning domains of two or more different CRF<sub>1</sub> molecules, as observed in other GPCRs (Guo et al., 2008). This possibility is also consistent with the reported ability of CRF<sub>1</sub> to oligomerize (Kraetke et al., 2005).

Simultaneous mutation to Ser of the three reactive Cys as well as Cys128 and Cys150 greatly decreased the sensitivity to MTSEA of the resulting receptor ( $\Delta$ Cys). There was a small amount of inhibition of  $\Delta$ Cys, despite the fact that the remaining Cys are thought to be disulfide cross-linked and therefore unreactive (Pioszak et al., 2008; Qi et al., 1997). It is possible that there is partially incomplete disulfide bonding when the receptor is expressed heterologously, which could lead to a small inhibition of sauvagine

binding to  $\Delta$ Cys by MTSEA. Another possible explanation could be that MTSEA reacts with a CRF<sub>1</sub>-associated protein to allosterically inhibit radioligand binding to CRF<sub>1</sub>. Curiously, the amino-terminal extracellular region of CRF receptors, which is essential for radioligand binding, forms a Sushi domain, which has been implicated in protein-protein interactions (Perrin et al., 2006).

Substitution of Ser for Cys211, Cys233 or Cys364 rendered the receptor significantly less sensitive to MTSEA. This suggests that no single endogenous Cys, was sufficient, after its reaction with MTSEA, to reduce sauvagine binding to CRF<sub>1</sub>. Consistent with this interpretation, reaction of MTSEA with the mutants  $\Delta$ Cys+211C,  $\Delta$ Cys+233C and  $\Delta$ Cys+364C, which contain only one of the reactive endogenous Cys, failed to inhibit sauvagine binding. Interestingly, simultaneous addition of the three Cys ( $\Delta$ Cys+211C+233C+364C), but no combination of two, into  $\Delta$ Cys restored the WT sensitivity to MTSEA. This suggests that MTSEA reacted simultaneously with Cys211, Cys233, and Cys364, to disrupt binding of Tyr<sup>0</sup>-sauvagine.

The small non-peptide CRF<sub>1</sub>-selective antagonist, antalarmin, protected CRF<sub>1</sub> against MTSEA reaction. Antalarmin has been proposed to bind to the TMs of CRF<sub>1</sub> (Hoare et al., 2003; Liaw et al., 1997). Given that the reactive Cys211, Cys233 and Cys364 are expected to be deep within the crevice near the cytoplasmic ends of their TMs, it is likely that antalarmin protected them from MTSEA reaction by binding above them and blocking the passage of reagent from the extracellular medium to the cytoplasmic end of the crevice. It is less likely that this non-peptide CRF analog reached the cytoplasmic ends of TMs of CRF<sub>1</sub> and directly protected Cys211, Cys233 and Cys364 from MTSEA reaction; ligands for different GPCRs and with smaller size than

antalarmin, such as epinephrine, dopamine and acetylcholine, have been shown to bind to residues located approximately in the middle of the TMs of their receptors (Fu et al., 1996; Liapakis et al., 2000; Pollock et al., 1992; Strader et al., 1988; Ward et al., 1999). Similarly, the D2 dopamine receptor (D2R) antagonist sulpiride protected Cys substituted for Ser129 at the cytoplasmic end of TM3 and for Val378 at the cytoplasmic end of TM6 from reaction with MTSEA by binding more extracellularly (Javitch et al., 1998; Javitch et al., 1995). These findings are consistent with the inability of the bulkier MTSET, to reach the endogenous TM Cys of CRF<sub>1</sub>. In addition to the possibility of protection through blocking passage of reagent, we cannot rule out an indirect protection through a ligand-mediated propagated structural rearrangement.

In contrast to our results, theoretical arrangements of the seven helices of family B GPCRs deduced from a detailed analysis of their sequences placed the residues of GLP-1 receptor that correspond to Cys211, Cys233 and Cys364 of CRF<sub>1</sub> facing lipid (Donnelly, 1997; Frimurer and Bywater, 1999). Similarly, in the family A GPCRs, D2 dopamine and rhodopsin receptor, residues 3.48, and 4.47, which are predicted to be aligned with Cys211 and Cys233 of CRF<sub>1</sub>, respectively (Frimurer and Bywater, 1999), face away from the binding-site crevice (Baldwin, 1993; Ballesteros et al., 2001; Palczewski et al., 2000). It is conceivable that these cysteines are located at a water-accessible interface of a CRF<sub>1</sub> oligomeric complex. However, it is also possible that in a single CRF<sub>1</sub> molecule local distortions, such as those induced by the presence of Pro and/or Gly above Cys211 and Cys233, could alter the configuration of TM3 and TM4 such as to position these Cys facing into the protein interior where they are accessible to MTSEA. Consistent with this proposal, Pro and Gly are known to modulate alpha-helical



structure (Deupi et al., 2005). Similarly, the presence of a Gly a few residues above Cys364 might alter the conformation of TM7 to position this Cys facing into the protein interior. Residue 7.54 of the  $\beta$ 2-adrenergic and D2 dopamine receptor, which are predicted to be aligned with Cys364 of CRF<sub>1</sub>, has been shown to be accessible for reaction with MTSEA (Fu et al., 1996; Liapakis and Javitch, 1998). This irregular pattern of accessibility has been proposed to be closely associated with a proline kink (Pro kink) at Pro7.50, which is conserved in family A GPCRs, in agreement with the crystal structure of rhodopsin (Ballesteros et al., 2001; Fu et al., 1996; Palczewski et al., 2000). The theoretical models of family B GPCRs, therefore, will need to be refined based on experimental data, including those of the present study. This is further supported by the observed inconsistencies between these theoretical models and the experimental data from a study on parathyroid hormone receptor, which determined the distances between His at the cytoplasmic ends of TM3 and TM6, by their ability to form zinc bridges (Sheikh et al., 1999).

Starting with the MTSEA-insensitive  $\Delta$ Cys mutant of CRF<sub>1</sub>, we can now systematically replace with Cys the amino acids of the membrane-spanning segments of CRF<sub>1</sub> and apply the substituted-cysteine accessibility method (SCAM) to characterize the residues lining the binding-site crevice. The resulting data will be used to refine theoretical models of family B GPCRs, ultimately advancing structure-based rational drug design.

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## LEGENDS FOR FIGURES

**Fig.1.** Effects of MTS reagents on specific [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine binding to CRF<sub>1</sub>. Suspensions of HEK 293 cells stably expressing the wild type CRF<sub>1</sub> were incubated for 15 sec at 22-25°C without (control) or with 2.5 mM of the MTS reagents, MTSEA, MTSET or MTSES. Subsequently the cells were homogenized and membrane homogenates were assayed for specific binding with [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine, as described under Materials and Methods. The bars represent the specific binding (% of control), or residual binding, which is defined as the percentage of [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine specific binding to MTS-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean  $\pm$  S.E values are from 8-40 independent experiments, each performed with duplicate determinations. The asterisk indicates that the MTSEA significantly decreased [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine specific binding to CRF<sub>1</sub> treated with MTSEA compared to the untreated receptor ( $P < 0.05$ , one-way analysis of variance and least significant difference post hoc test).

**Fig.2.** Dose dependent effect of MTSEA on specific binding of [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine to CRF<sub>1</sub>. Suspensions of HEK 293 cells stably expressing the wild type CRF<sub>1</sub> were incubated for 15 sec at 22-25 °C without (control) or with various concentrations of MTSEA. Subsequently the cells were homogenized and membrane homogenates were assayed for specific binding with [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine, as described under Materials and Methods. Means  $\pm$  S.E are shown from 3-40 independent experiments, each performed with duplicate determinations.

**Fig.3.** Effect of MTSEA reaction on the binding properties of CRF<sub>1</sub>. Suspensions of HEK 293 cells stably expressing the wild type CRF<sub>1</sub> were treated without (control) or with 2.5 mM or 15 mM MTSEA for 15 sec at 22-25 °C. Subsequently the cells were homogenized and membrane homogenates were incubated with 20-25 pM [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine in the absence or presence of increasing concentrations of Tyr<sup>0</sup>-sauvagine. The data were fit to a one-site competition model by nonlinear regression and -logK<sub>D</sub> values were determined as described under Materials and Methods. The bars represent the binding affinity (-logK<sub>D</sub>) of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine for CRF<sub>1</sub> before or after its treatment with MTSEA. The mean ± S.E. values are from 2-6 independent experiments. The affinity of radioligand for the untreated receptor was not statistically different from that for the CRF<sub>1</sub> treated with either 2.5 mM or 15 mM MTSEA (one-way ANOVA followed by least significant difference post hoc test).

**Fig.4.** Protection of [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine specific binding by preincubation with varying concentrations of antalarmin. HEK 293 cells stably expressing the wild type CRF<sub>1</sub> were preincubated with increasing concentrations (1-1000 nM) of the antagonist, antalarmin, for 30 min at 37°C and subsequently treated with 2.5 mM MTSEA as described under Materials and Methods. Subsequently the cells were homogenized and the ability of membrane homogenates to bind [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine was assayed as described under Materials and Methods. The dotted line represents the specific binding after treatment with 2.5 mM MTSEA in the absence of antalarmin. The specific binding (% of control) was defined as specific binding of [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine to MTSEA-treated CRF<sub>1</sub> divided

by the specific binding to the untreated receptors (control). Means  $\pm$  S.E are shown from six independent experiments, each performed with duplicate determinations.

**Fig.5.** Snake plot representation of CRF<sub>1</sub> showing the approximate positions of its 13 cysteines. The cylinders represent the membrane-spanning segments (TM1-TM7) of CRF<sub>1</sub>. Four endogenous cysteines (Cys128, Cys211, Cys233 and Cys364) are located in the membrane-spanning segments of CRF<sub>1</sub>, whereas one is positioned in the first intracellular loop (Cys150) of receptor. Six endogenous cysteines are located in the extracellular N-region (Cys30 and Cys54, Cys44 and Cys87, and Cys68 and Cys102), forming three disulfide bridges (dotted lines) (Pioszak et al., 2008). The resting two endogenous cysteines (Cys188 and Cys258) are positioned in the first and second extracellular loops of CRF<sub>1</sub>, and form a disulfide bond (dotted line) (Qi et al., 1997).

**Fig. 6.** Binding affinity of Tyr<sup>0</sup>-sauvagine for C128S, C211S, C233S and C364S receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing wild type (WT) CRF<sub>1</sub> or the mutants, C128S, C211S, C233S, or C364S were treated without (control) or with 2.5 mM MTSEA for 15 sec at 22-25 °C. Subsequently the cells were homogenized and membrane homogenates were incubated with [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine in the absence or presence of increasing concentrations of Tyr<sup>0</sup>-sauvagine. The data were fit to a one-site competition model by nonlinear regression and -logK<sub>D</sub> values were determined as described under Materials and Methods. The bars represent the binding affinity (-logK<sub>D</sub>) of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine before (open bars) or after MTSEA reaction (filled bars). The mean  $\pm$  S.E. values are from 2–6 independent experiments. The

results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations significantly altered [ $^{125}$ I]-Tyr<sup>0</sup>sauvagine affinity and MTSEA treatment did not significantly alter radioligand affinity of any of the receptors tested.

**Fig. 7.** Effects of MTSEA reaction on specific [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine binding to C128S, C211S, C233S, or C364S receptors. Suspensions of HEK 293 cells stably expressing wild type CRF1 (WT) or C128S, C211S, C233S, C364S mutants were incubated for 15 sec at 22-25 °C with or without (control) 2.5 mM MTSEA. Subsequently the cells were homogenized, and membrane homogenates were assayed for specific binding with [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine, as described under Materials and Methods. The bars represent the S.B (% of control), or residual binding, which is defined as the percentage of [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean  $\pm$  S.E values are from 9-40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to C211S, C233S or C364S mutant after MTSEA reaction was significantly different from the corresponding one to WT ( $P < 0.05$ , one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to C211S, C233S and C364S mutants after MTSEA reaction was not statistically different from each other.

**Fig. 8.** Binding affinity of Tyr<sup>0</sup>-sauvagine for  $\Delta$ Cys,  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C, and  $\Delta$ Cys + 364C receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing wild type (WT) CRF<sub>1</sub> or the mutants,  $\Delta$ Cys,  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C, or  $\Delta$ Cys + 364C were treated without (control) or with 2.5 mM MTSEA for 15 sec at 22-25 °C. Subsequently the cells were homogenized and membrane homogenates were incubated with [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine in the absence or presence of increasing concentrations of Tyr<sup>0</sup>-sauvagine. The data were fit to a one-site competition model by nonlinear regression and -logK<sub>D</sub> values were determined as described under Materials and Methods. The bars represent the binding affinity (-logK<sub>D</sub>) of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine before (open bars) or after MTSEA reaction (filled bars). The mean  $\pm$  S.E. values are from 2-6 independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations considerably altered [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine affinity and MTSEA treatment of all receptors tested did not significantly alter radioligand affinity.

**Fig. 9.** Effects of MTSEA reaction on specific [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine binding to  $\Delta$ Cys,  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C, or  $\Delta$ Cys + 364C receptors. Suspensions of HEK 293 cells stably expressing wild type CRF<sub>1</sub> (WT) or  $\Delta$ Cys,  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C, or  $\Delta$ Cys + 364C mutants were incubated for 15 sec at 22-25 °C with or without (control) 2.5 mM MTSEA. Subsequently the cells were homogenized, and membrane homogenates were assayed for specific binding with [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine, as described under Materials and Methods. The bars represent the S.B (% of control), or residual binding, which is defined as the percentage of [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine specific

binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean  $\pm$  S.E values are from 8-40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to  $\Delta$ Cys,  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C, or  $\Delta$ Cys + 364C mutant after MTSEA reaction was significantly different from the corresponding one to WT ( $P < 0.05$ , one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to  $\Delta$ Cys,  $\Delta$ Cys + 128C,  $\Delta$ Cys + 211C,  $\Delta$ Cys + 233C and  $\Delta$ Cys + 364C mutants after MTSEA reaction was not statistically different from each other.

**Fig. 10.** Binding affinity of Tyr<sup>0</sup>-sauvagine for  $\Delta$ Cys + 211C + 233C,  $\Delta$ Cys + 211C + 364C,  $\Delta$ Cys + 233C + 364C, and  $\Delta$ Cys + 211C + 233C + 364C receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing wild type (WT) CRF<sub>1</sub> or the mutants,  $\Delta$ Cys,  $\Delta$ Cys + 211C + 233C,  $\Delta$ Cys + 211C + 364C,  $\Delta$ Cys + 233C + 364C, or  $\Delta$ Cys + 211C + 233C + 364C were treated without (control) or with 2.5 mM MTSEA for 15 sec at 22-25 °C. Subsequently the cells were homogenized and membrane homogenates were incubated with [<sup>125</sup>I] Tyr<sup>0</sup>-sauvagine in the absence or presence of increasing concentrations of Tyr<sup>0</sup>-sauvagine. The data were fit to a one-site competition model by nonlinear regression and -logK<sub>D</sub> values were determined as described under Materials and Methods. The bars represent the binding affinity (-logK<sub>D</sub>) of [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine before (open bars) or after MTSEA reaction (filled bars). The mean  $\pm$  S.E. values are from 2-6 independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of

the mutations considerably altered [ $^{125}$ I]-Tyr<sup>0</sup>-sauvagine affinity and MTSEA treatment did not significantly alter radioligand affinity of the receptors tested.

**Fig. 11.** Effects of MTSEA reaction on specific [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine binding to  $\Delta$ Cys + 211C + 233C,  $\Delta$ Cys + 211C +364C,  $\Delta$ Cys + 233C +364C, or  $\Delta$ Cys + 211C + 233C +364C receptors. Suspensions of HEK 293 cells stably expressing wild type CRF<sub>1</sub> (WT) or  $\Delta$ Cys,  $\Delta$ Cys + 211C + 233C,  $\Delta$ Cys + 211C +364C,  $\Delta$ Cys + 233C +364C, or  $\Delta$ Cys + 211C + 233C +364C mutants were incubated for 15 sec at 22-25 °C with or without (control) 2.5 mM MTSEA. Subsequently the cells were homogenized, and membrane homogenates were assayed for specific binding with [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine, as described under Materials and Methods. The bars represent the S.B (% of control), or residual binding, which is defined as the percentage of [ $^{125}$ I] Tyr<sup>0</sup>-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean  $\pm$  S.E. values are from 3–40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to  $\Delta$ Cys,  $\Delta$ Cys + 211C + 233C,  $\Delta$ Cys + 211C +364C or  $\Delta$ Cys + 233C +364C mutant after MTSEA reaction was significantly different from the corresponding one to WT or to  $\Delta$ Cys + 211C + 233C +364C ( $P < 0.05$ , one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to  $\Delta$ Cys,  $\Delta$ Cys + 211C + 233C,  $\Delta$ Cys + 211C +364C, or  $\Delta$ Cys + 233C +364C mutants after MTSEA reaction was not statistically different from each other. In addition, the residual binding to WT and to  $\Delta$ Cys + 211C + 233C +364C after MTSEA reaction was not statistically different from each other.



Fig. 1

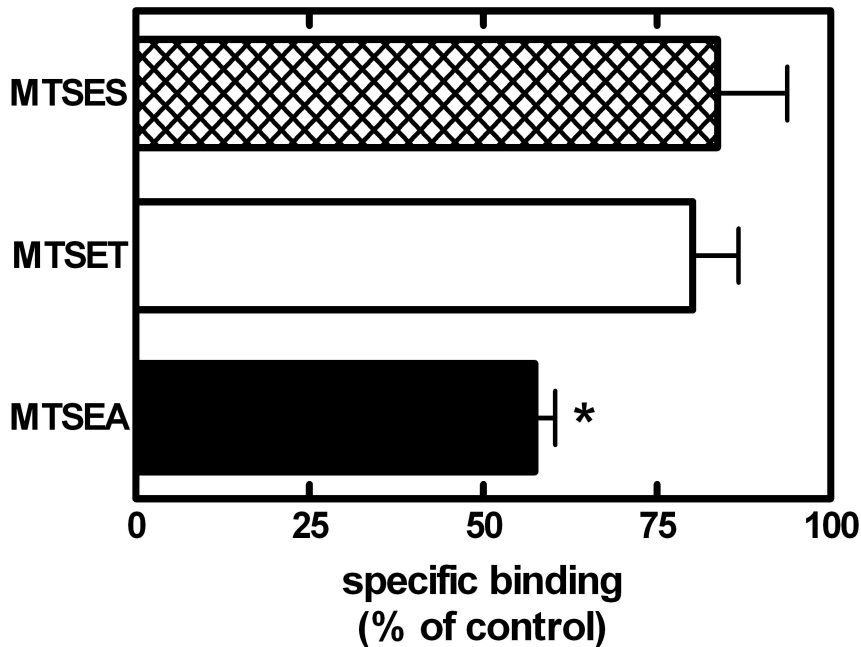


Fig. 2

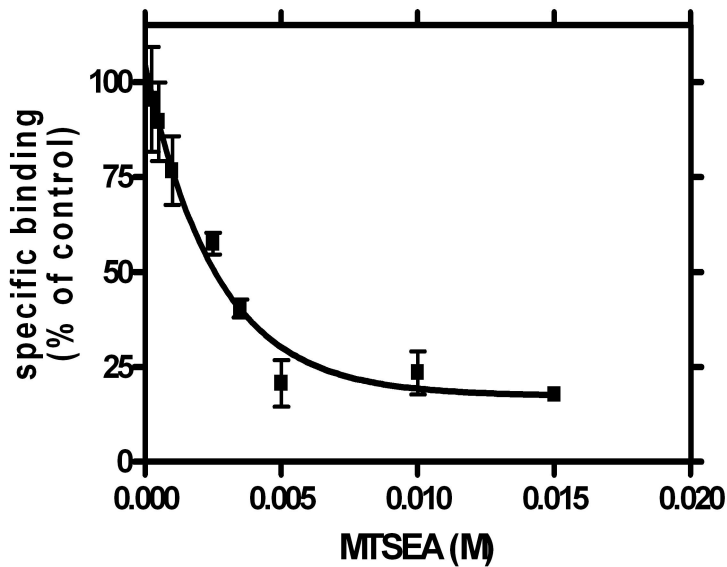


Fig. 3

- (LogK<sub>D</sub> ± S.E.)

8.94 ± 0.04
9.11 ± 0.13
9.05 ± 0.08

MTSEA 15 mM

MTSEA 2.5 mM

CONTROL

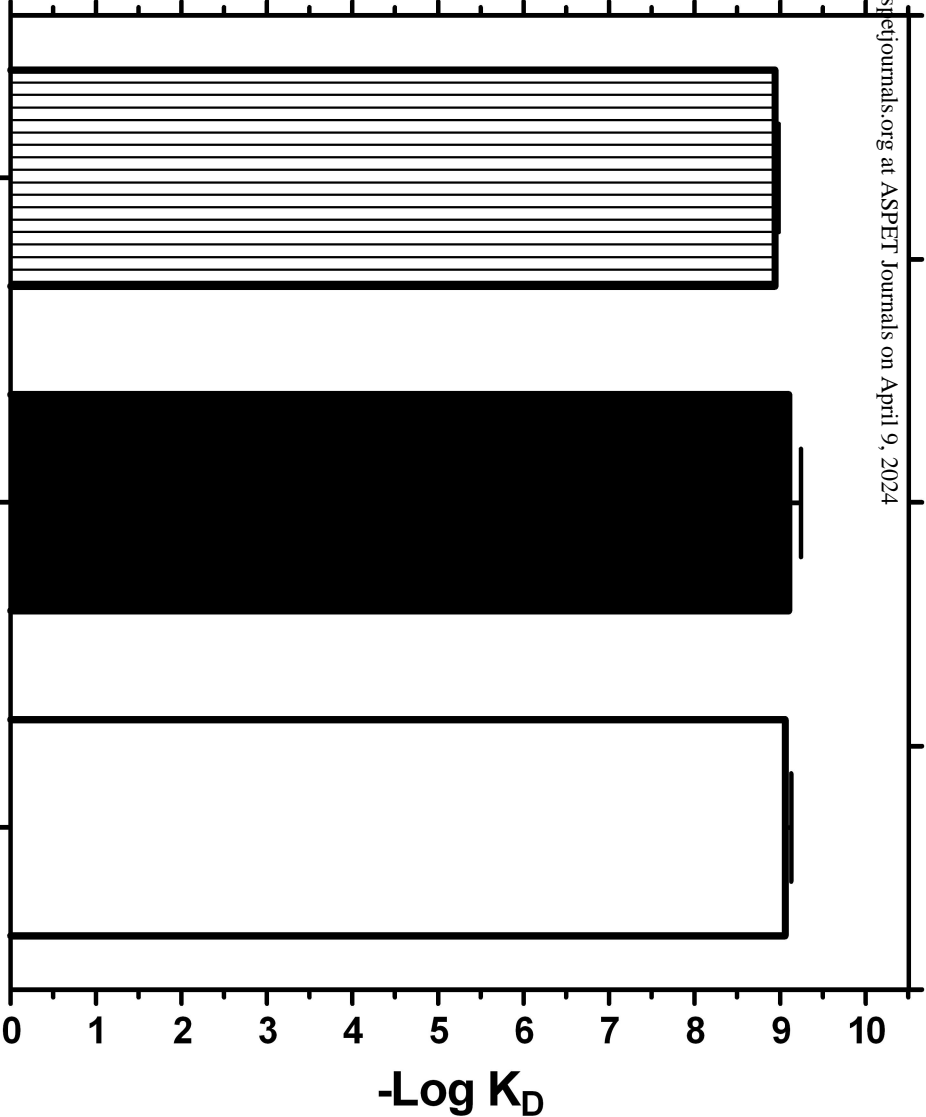
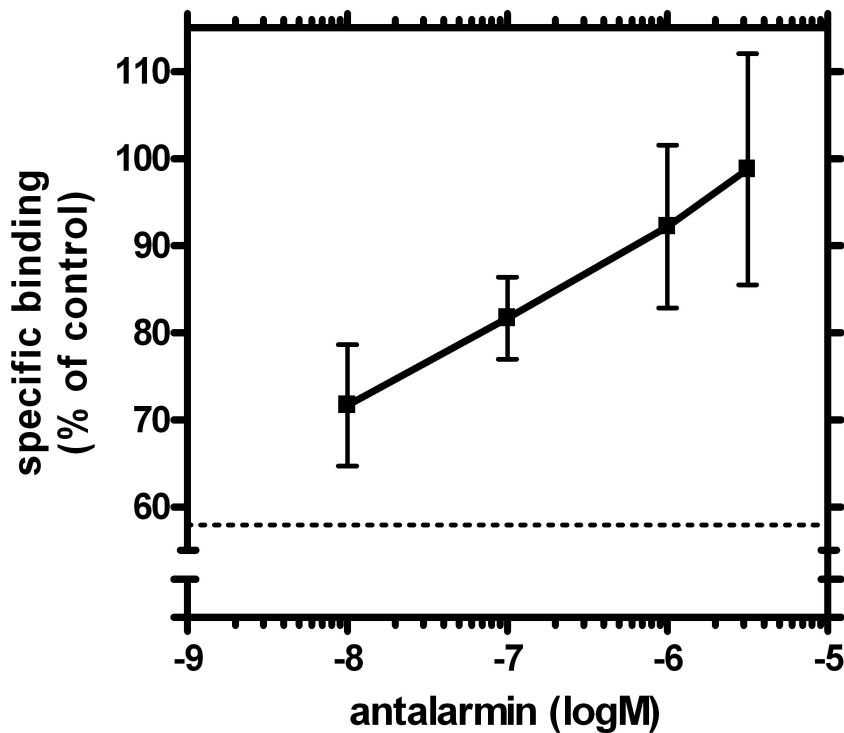


Fig. 4



**Fig. 5**

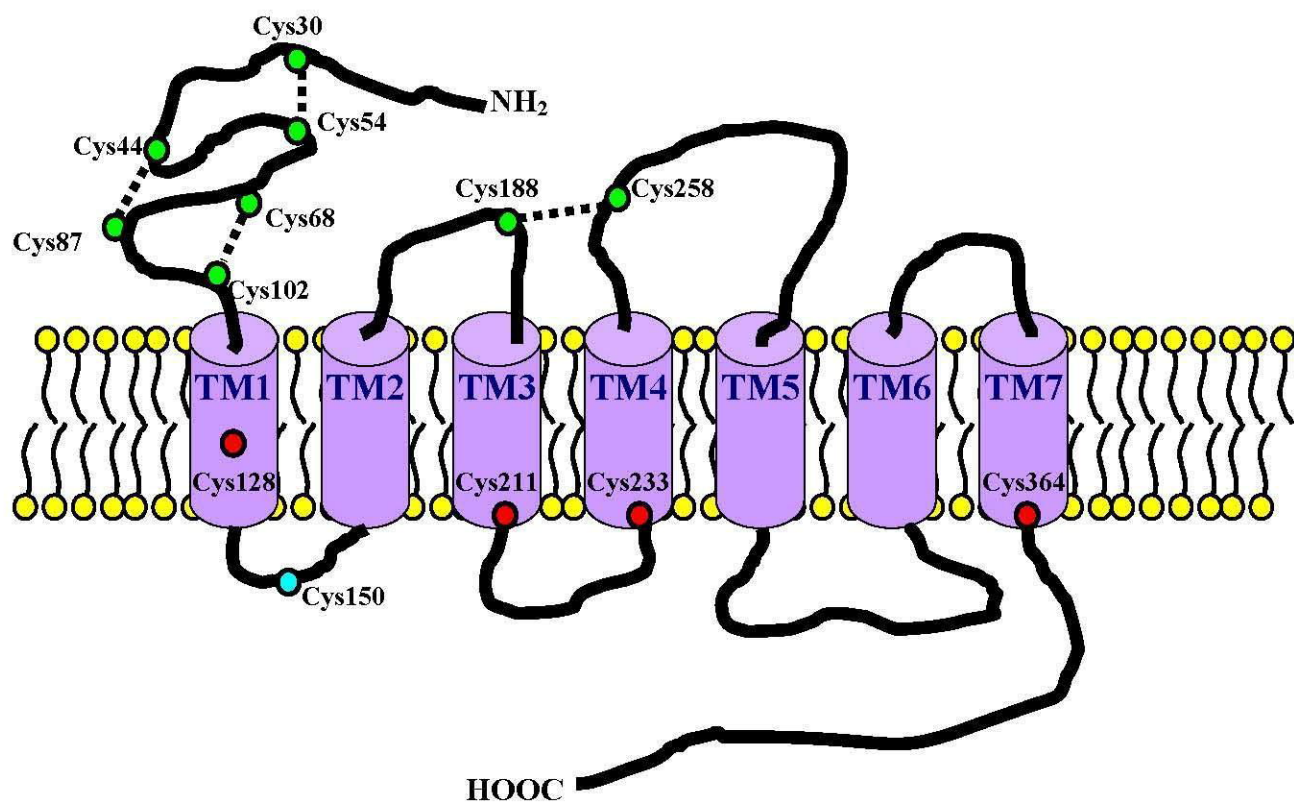


Fig. 6

- (LogK <sub>D</sub> ± S.E.)	
CONTROL	MTSEA
8.59 ± 0.18	8.49 ± 0.16
9.04 ± 0.15	8.67 ± 0.14
8.98 ± 0.07	9.03 ± 0.03
8.45 ± 0.15	8.37 ± 0.20
9.05 ± 0.08	9.11 ± 0.13

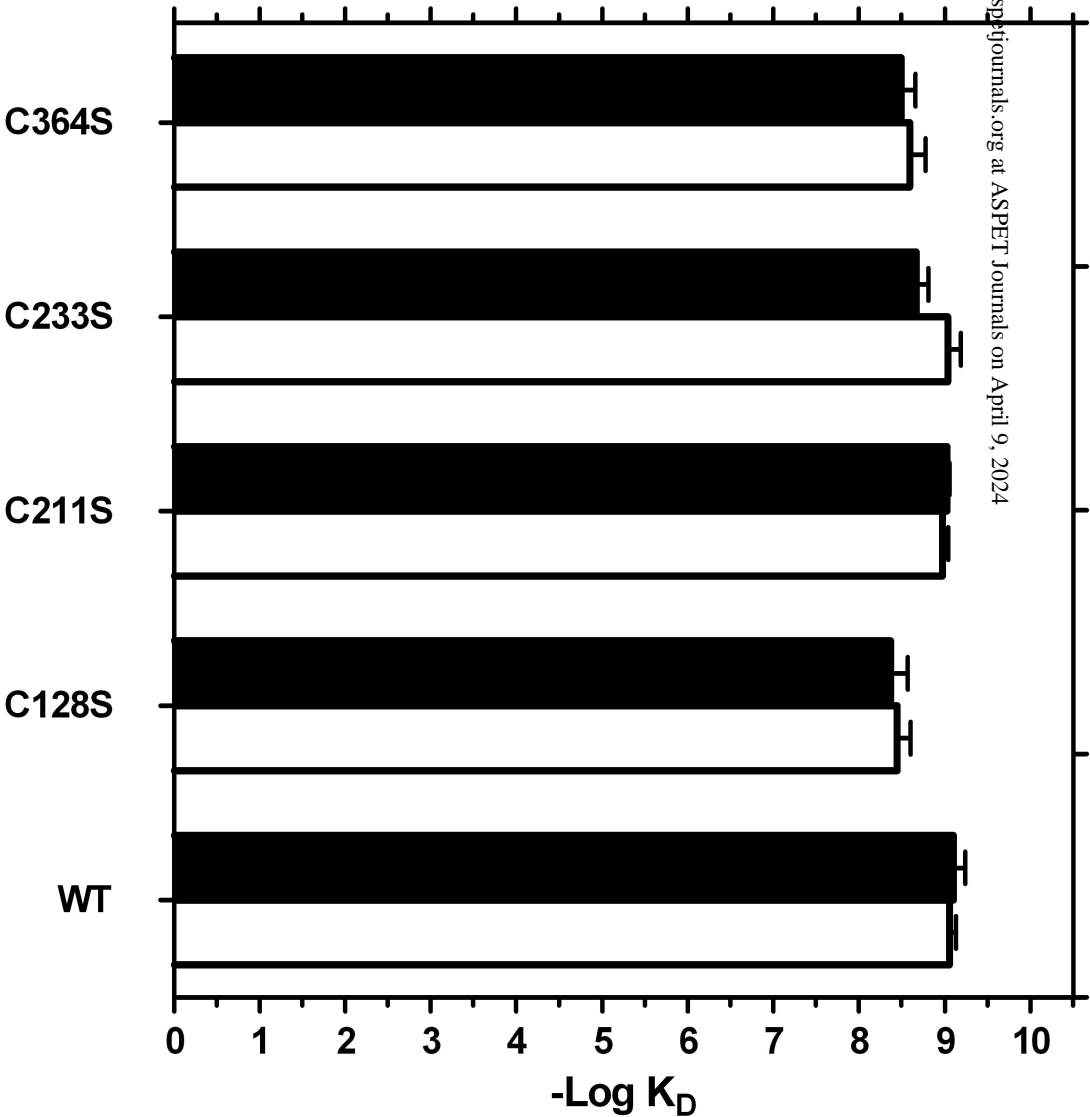


Fig. 7

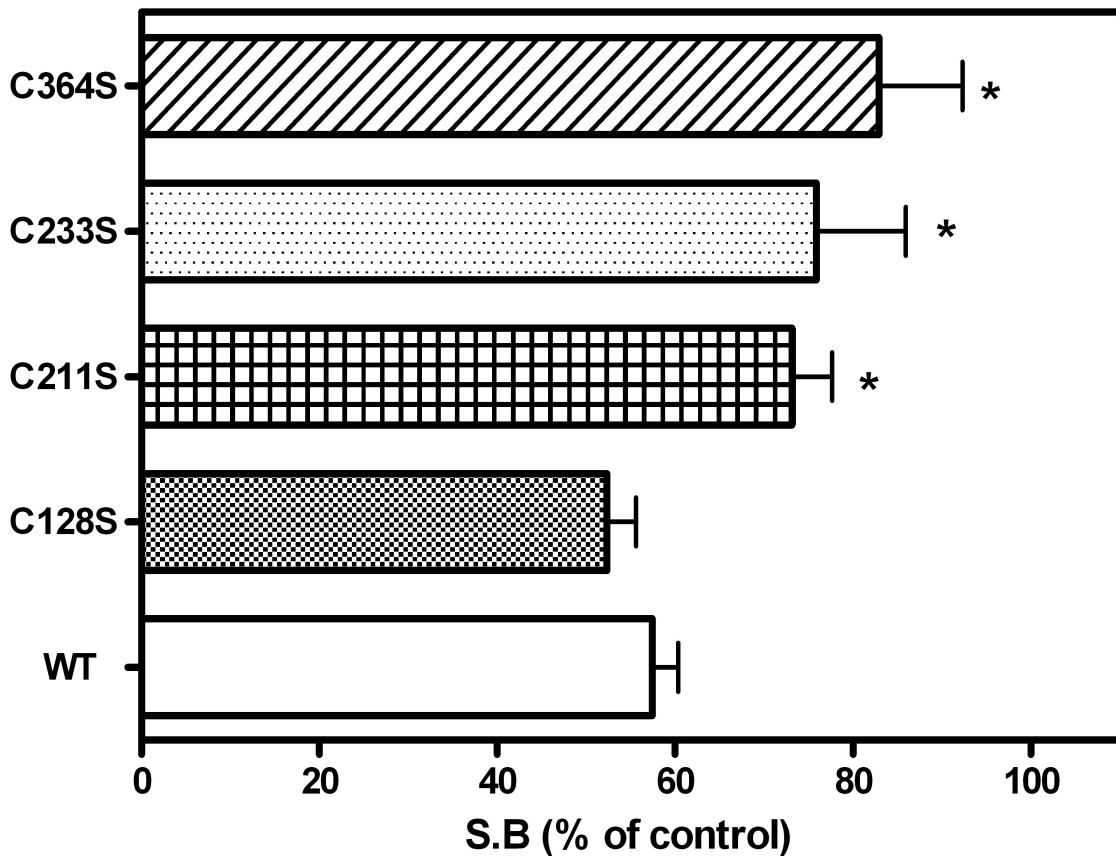


Fig. 8

- (LogK<sub>D</sub> ± S.E.)

CONTROL	MTSEA
8.68 ± 0.60	8.51 ± 0.89
8.79 ± 0.42	8.53 ± 0.38
8.79 ± 0.50	8.34 ± 0.48
8.52 ± 0.40	8.26 ± 0.18
8.60 ± 0.63	8.60 ± 0.54
9.05 ± 0.08	9.11 ± 0.13

ΔCys+364C

ΔCys+233C

ΔCys+211C

ΔCys+128C

ΔCys

WT

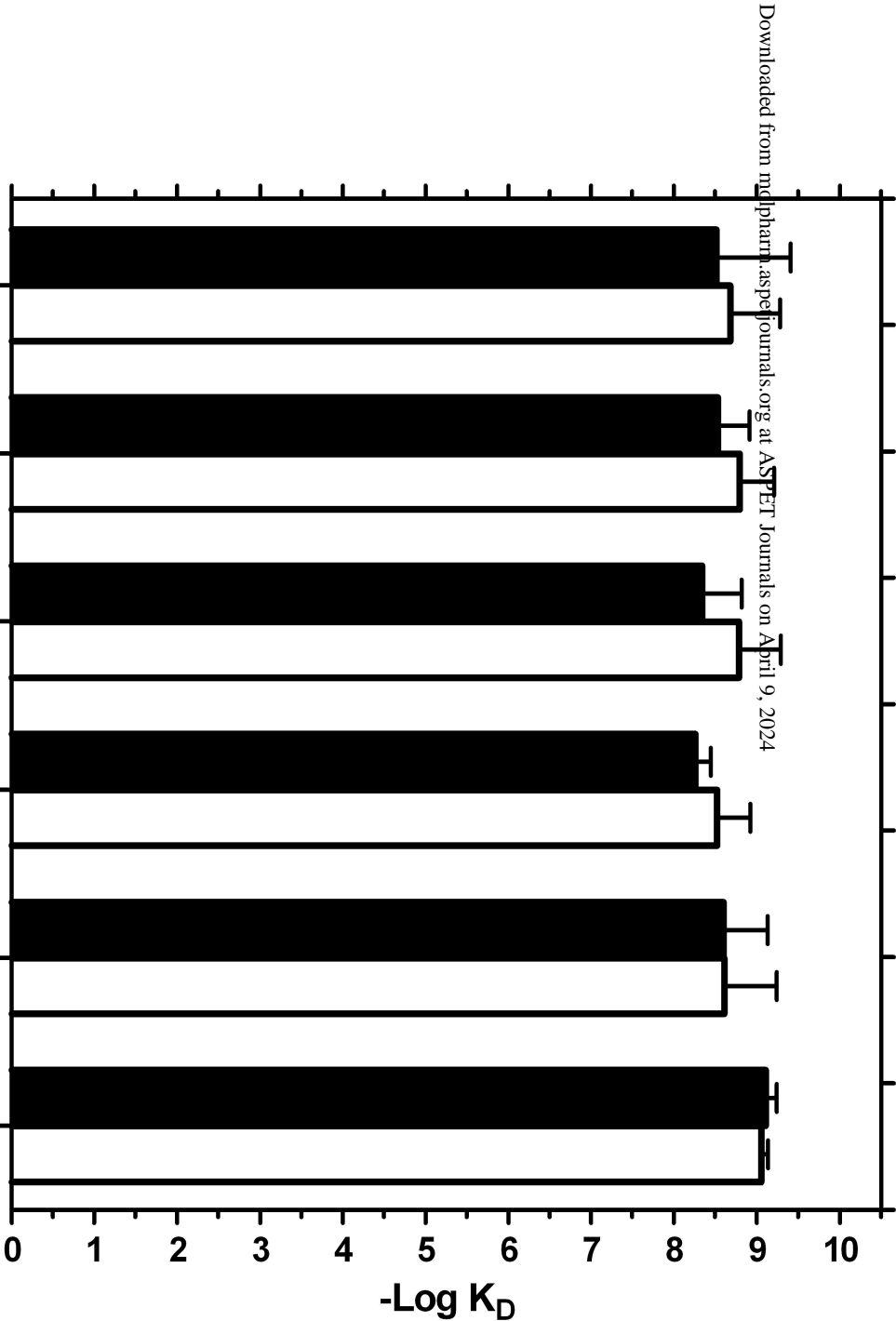




Fig. 9

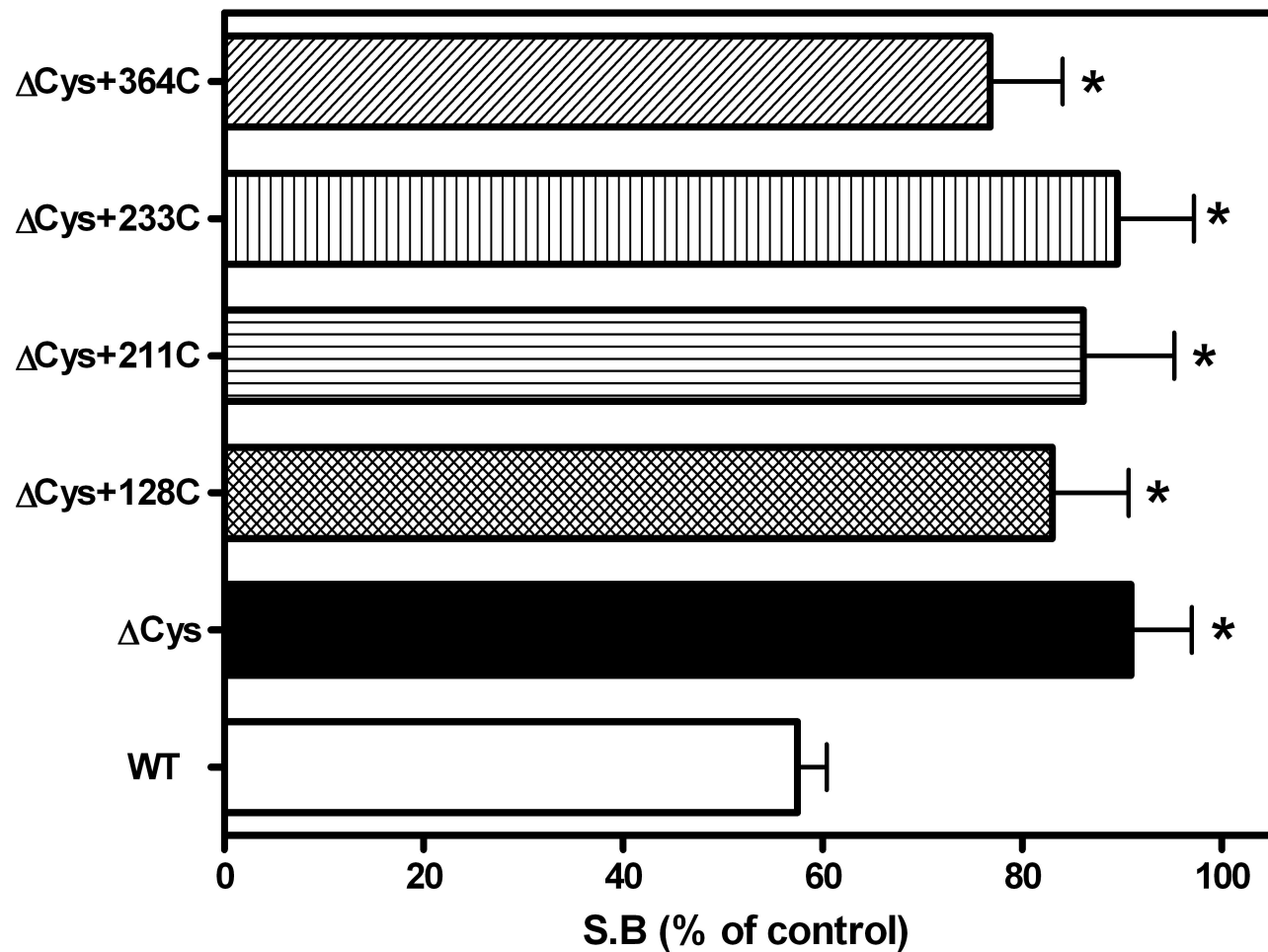


Fig. 10

- (LogK <sub>D</sub> ± S.E.)	
CONTROL	MTSEA
8.85 ± 0.10	8.80 ± 0.13
9.11 ± 0.05	8.89 ± 0.04
9.11 ± 0.09	8.88 ± 0.08
8.87 ± 0.16	8.80 ± 0.23
8.60 ± 0.63	8.60 ± 0.54
9.05 ± 0.08	9.11 ± 0.13

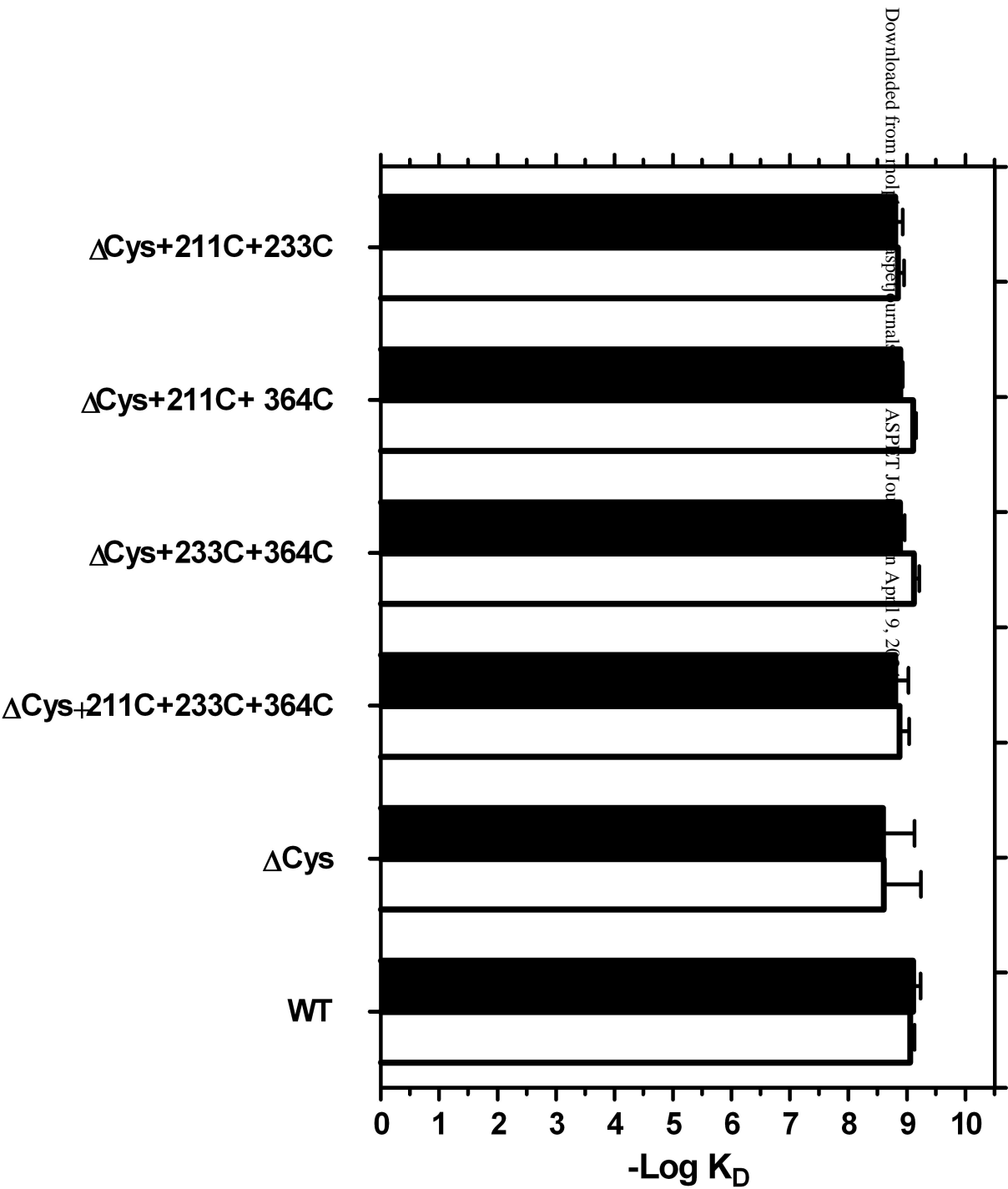


Fig. 11

