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Title Page

Adenosine A_{2A} Receptor Stimulation Increases Angiogenesis By Downregulating Production Of The Anti-Angiogenic Matrix Protein Thrombospondin 1 (TSP1)

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Running Title Page

A_{2A} receptor downregulation of TSP1

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Discussion: 937 words

Abbreviations: CGS-21680, 2-p-[2-carboxyethyl] phenethyl-amino-5'-N-ethylcarboxamido-adenosine; MRE0094, 2-[2-(4-Chlorophenyl) ethoxy]adenosine; ZM241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl}phenol; DPCPX, diphenylcyclopentylxanthine; MRS 1706, *N*-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide; HMVEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells.

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ABSTRACT

Topical adenosine A_{2A} receptor agonists promote wound healing by, among other effects, increasing microvessel formation. Results of representational display analysis of human umbilical vein endothelial cells suggested that A_{2A} receptor occupancy modulates expression of the anti-angiogenic matrix protein thrombospondin 1 (TSP1). We therefore determined whether A_{2A} receptor occupation stimulates angiogenesis by modulating TSP1 secretion. Human microvascular endothelial cells (HMVEC) were treated with medium alone, CGS-21680 or MRE0094, selective A_{2A} receptor agonists. TSP1 protein secretion was downregulated following treatment with the A_{2A} agonists CGS-21680 or MRE0094 in a dose-dependent manner (EC50=6.65 nM or 0.23 μM, respectively). The selective A_{2A} receptor antagonist ZM241385 but not the A1 and A_{2B} receptor antagonists DPCPX, enprofylline and MRS1706 completely abrogated the A_{2A} receptor agonist-mediated effect on TSP1. Vascular tube formation by HMVEC was increased by adenosine A_{2A} receptor agonists in a dose-dependent fashion (EC50= 0.1 μM for both) and this effect was reversed by the A_{2A} antagonist. Moreover, in the presence of antibodies to TSP1 and CD36, the receptor for TSP1, the adenosine A_{2A} receptor agonists stimulated no increase in vascular tube formation. These results indicate that the angiogenic effects of adenosine A_{2A} receptor activation are, at least in part, due to suppression of TSP1 secretion.

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INTRODUCTION

Angiogenesis, the formation of new blood vessels from a pre-existing vasculature, is tightly regulated in normal adults. Growth of new capillaries is controlled by interplay of growth regulatory factors which either stimulate or inhibit blood vessel growth (Medina et al., 2004; Nicholson and Theodorescu, 2004; Westphal et al., 2000). Many angiogenic factors have been described, including vascular endothelial growth factor, basic fibroblast growth factor, and thymidine phosphorylase. More recently, a number of naturally occurring inhibitors of angiogenesis, including thrombospondin and angiostatin, have also been identified (Liu et al., 1999; Westphal et al., 2000).

Adenosine, a potent endogenous physiological mediator, regulates a wide variety of physiological processes. Adenosine mediates its physiological effects via interaction with one or more of four G protein coupled receptors (A_1, A_{2A}, A_{2B}, and A_3), expressed on many cell types including neutrophils, macrophages, fibroblasts and endothelial cells (Montesinos et al., 2002; Montesinos et al., 2003; Montesinos et al., 1997; Montesinos et al., 2000; Victor-Vega et al., 2002). Recent studies in wild type and adenosine A_{2A} receptor knockout mice demonstrate that adenosine A_{2A} receptor occupancy stimulates angiogenesis and increases the rate at which wounds close (Montesinos et al., 2002; Montesinos et al., 1997). Adenosine A_{2A} receptor-mediated regulation of angiogenic factors, such as VEGF (Feoktistov et al., 2002; Leibovich et al., 2002; Montesinos et al., 2002; Nguyen et al., 2003; Olah and Caldwell, 2003), plays an important role in adenosine A_{2A} receptor-stimulated angiogenesis in healing wounds but adenosine A_{2A} receptors may modulate other functions as well.

We report here that Representational Display Analysis of medium- or CGS-21680 (an A_{2A} receptor agonist)-stimulated human umbilical vein endothelial cells identified adenosine A_{2A}

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receptor-mediated modulation of the anti-angiogenic factor thrombospondin 1 (TSP1) as another potential contributor to A_{2A} receptor-stimulated angiogenesis. In support of this hypothesis we found that adenosine A_{2A} receptor agonists suppressed TSP1 mRNA and protein expression. Studies of vascular tube formation on matrigel further demonstrate that adenosine A_{2A} receptor-mediated suppression of TSP1 expression plays an important role in adenosine A_{2A} receptor-mediated enhancement of angiogenesis.

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MATERIALS AND METHODS

Materials: CGS-21680 (2-p-[2-carboxyethyl] phenethyl-amino-5'-N-ethylcarboxamido-adenosine), was obtained from Sigma Chemical Co. (St. Louis, MO) and MRE0094 (2-[2-(4-Chlorophenyl) ethoxy) adenosine, was a generous gift of King Pharmaceuticals (Research Triangle Park, NC). ZM241385, a highly selective A_{2A} receptor antagonist (Tocris; Ballwin, MO), DPCPX, an A_1 receptor antagonist (Sigma; St. Louis, MO) MRS1706, a more potent and selective A_{2B} receptor antagonist (Tocris; Ballwin, MO), and enprofylline, a selective A_{2B} receptor antagonist (Sigma; St. Louis, MO) were used in this study. TSP1 protein was obtained from Sigma Chemical Co. (St. Louis, MO). All other materials were of the highest quality that could be obtained.

Cell Culture: Human microvascular endothelial cells (Cambrex; Walkersville, MD) were grown in 6 well plates with fully supplemented EBM-2MV medium (Cambrex; Walkersville, MD) to 80-90% confluence. HMVEC were incubated with A_{2A} receptor agonist CGS-21680 or MRE0094 at concentrations ranging from $1x10^{-9}$ M to $1x10^{-5}$ M (24hrs, 37°C and 5% CO_2) with or without the addition of adenosine receptor antagonists DPCPX (A_1), ZM241385 (A_{2A}), enprofylline (A_{2B}); each at 10μM. Additional experiments were also performed using ZM241385 (10nM) and MRS 1706 (10nM). Supernates were collected for western blots and mRNA was isolated for RT-PCR, real time PCR and microchip array analysis.

RNA Isolation: Total RNA was extracted from the treated and untreated HMVEC cell line using Trizol Reagent (Gibco; Carlsbad, CA). Residual genomic DNA was removed by incubating the RNA with DNAse (Ambion; Austin, Texas). The quantity of total RNA from each condition was measured by Hitachi U2010 spectrophotometer (Hitachi; Brisbane, CA). The quality of the RNA was verified by gel electrophoresis. The total RNA yield from $1x10^6$ cultured cells was 8-15μg.

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Reverse Transcription: All reverse transcription (RT) reactions and polymerase chain reactions (PCR) were carried out in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer; Branchburg, NJ). For each assay, 1µg of mRNA was reverse transcribed using 50 U of Murine Leukemia Virus reverse transcriptase (Applied Biosystems; Branchburg, NJ) in a final volume of 50µl. The reaction mixtures were incubated at 42^{o}C for forty-five minutes followed by inactivation at 95^{o}C for five minutes and finally cooled to 5^{o}C for five minutes.

Polymerase Chain Reaction: 5ul of cDNA was used for both TSP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR amplification. The upstream and downstream primers for TSP1 and GAPDH were: 5' CCTGATGGAGAATGCTGTCC 3' and 5' CACATCGGTTGTTGAGGCTA 3' (NYU CORE facility) and 5' TGAAGGTCGGAGTCAACGGATTTGGT 3' and 5' CATGTGGGCCATGAGGTCCACCAC 3' respectively (Sigma; St. Louis, MO). The PCR reaction was carried out using PCR kit (Applied Biosystems; Branchburg, NJ) and 0.15µM of each upstream and downstream primer per reaction. The PCR protocol included 40 cycles at 95°C for 1 minute, 55^{o}C for 1 minute and 72^{o}C for 1 minute for TSP1 and 94^{o}C for 45 seconds, 58^{o}C for 1 min and 72^{o}C for 1 minute for GAPDH.

PCR Product Confirmation: The PCR products (10µl/lane) were mixed with 1µl of 6X DNA loading buffer (Gibco; Carlsbad, CA) and loaded directly onto a 1% agarose gel. The GAPDH controls (10ul/lane) were loaded at two concentrations, 1 and 0.20 ug/ul of RNA. The PCR products were visualized and photographed under ultraviolet light (320 nm) using a Kodak trans-illuminator. The band intensities were measured with Kodak Digital Science 1D, version 2.0.3, after imaging with Kodak Digital Science Electrophoresis Documentation and Analysis System

120 (Kodak; Rochester, NY). All experimental results were normalized to the intensity of GAPDH.

Generation of a Subtraction Library: HUVEC were grown to 70% confluence in T75 flasks. The night before the experiment, cells were refed with fresh media. After 4 hours incubation of HUVEC with fully supplemented DMEM media (10%FBS, L-glutamine, penicillin-Streptomicine) in the presence or absence of 1μM selective adenosine A_{2A} receptor agonist, poly(A)$^+$ mRNA was isolated using a microFastTrack mRNA Isolation Kit (InVitrogen; San Diego, CA) according to instructions from the manufacturer. Then cDNA was prepared using a RiboClone® cDNA Synthesis System according to instructions from the manufacturer (Promega; Madison, WI). The subtraction library was generated using the cDNA representational difference analysis (RDA) developed by Chu and Paul (Chu C.C. and Paul W.E. (1998) Mol. Immunol. 35: 487-502). Briefly, cDNA was digested by *Sau*3AI (New England Biolabs; Beverly, MA,U.S.A.) at 37°C under conditions suggested by the manufacturer, followed by incubation at 65°C for 20 min to stop the reaction. Driver amplicon (from untreated cells) and tester amplicon (from CGS-21680 treated cells) were generated by annealing specific sets of adaptors to the digested cDNA and subsequent PCR amplification as previously described. Driver amplicon was then modified by reamplification with 5' biotinylated adaptor primer. We performed four rounds of subtractions in hybridization buffer (0.5 M Phosphate Buffer, 0.1% SDS, 5 mM EDTA) at 65°C after denaturation at 100°C for 5min. Driver-tester and driver-driver hybrids were removed by magnetic bead depletion (Dynabeads® M-280 Streptavidin, Dynal; Lake Success, NY). Tester-tester hybrids were then amplified using a primer specific for the adaptor sequence ligated on the tester strand. Amplified material was digested with *Sau*3AI, purified with Chromaspin-100 columns, ligated into *Bam*HI (NEB) site of pBluescriptSK$^+$ (Stratagene) and transformed into

DH5α bacteria (Life Technologies; Gaithiersburg, MD) on selective plates (Luria-Bertani medium with agar and 100μg/ml ampicillin supplemented with Xgal IPTG (ICN Biomedicals; Aurora, OH)). Individual transformants were analyzed for DNA insert by PCR. Products larger than 164 bp indicate an insert had been cloned. These subtraction clone inserts were sequenced from purified plasmid DNA, in the core sequencing facility at NYU School of Medicine. Sequences were analysed using Sequencher™ and compared to public databases by using BLAST service provided by National Center for Biotechnology Information (Bethesda, MD).

Southern Blot Analysis: For analysis of RDA amplicons, driver, tester and amplicon samples (~0.25 μg) were separated on a 2%agarose gel and Southern blotted to nylon membrane (Optitran, Schleicher and Schuell; Keene, NH) by upward capillary in 10× SSC buffer (1.5 M NaCl, 0.15Msodium citrate (pH 7.0)). After UV crosslinking with UVcrosslinker (Fisher Scientific; Pittsburgh, PA), membranes were prehybridized for 3 h at 65°C in prehybridization solution (Amersham Biosciences; Piscataway, NJ) and then hybridized overnight at 65°C to radioactive-labeled probe in hybridization solution (Amersham Biosciences; Piscataway, NJ). GAPDH and VEGF probes were generated by PCR from HUVEC cDNA using 5'-gacccttcattgacctcaac-3' forward and 5'-gaggggccatccacagtcttc-3' reverse primers for GAPDH, and 5'-cgccatcctgtgtgccc-3' forward and 5'-ctcaccgcctcggcttgtc-3' reverse primers for VEGF. Probes were labeled with [α-^{32}P]dCTP (3000 Cimmol, Amersham Biosciences; Piscataway, NJ) using an Oligo labeling Kit (Pharmacia Biotech; Piscataway, NJ). After hybridization, the membranes were washed twice in 1× SSC, 0.1% SDS at room temperature, twice in 1× SSC, 0.1% SDS at 65°C and twice in 0.1× SSC, 0.1% SDS at 65°C and air dried. Radioactivity was analyzed and quantitated by phosphorimager (Molecular Dynamics; Sunnyvale, CA). For differential screening, PCR products of inserts from individual subtraction clones (~0.25μg)

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were electrophoresed in 2% agarose gels and then treated as above. Membranes were probed as above with [α-^{32}P]dCTP labeled driver or tester amplicon. Intensity of hybridization signal between tester and driver amplicon probes on each individual PCR product was compared by densitometric measurements of bands using Kodak Digital Science software.

Northern Blot Analysis: HMVEC were incubated with adenosine receptor agonists as described above. As previously described, denatured RNA samples (10μg) were electrophoresed in 1% agarose/formaldehyde gels and Northern blotted to supported nitrocellulose membrane (Optitran, Schleicher and Schuell; Keene, NH) by downward capillary method in 20X SSC (0.15 M NaCl plus 0.015 M sodium citrate). There after the membrane was rinsed in 2X SSC, air dried and UV crosslinked with UVcrosslinker (Fisher Scientific; Pittsburgh, PA), prehybridized for 3 h at 65°C in prehybridization solution (Amersham Biosciences; Piscataway, NJ) and then probed with [α-^{32}P]dCTP (3000 Cimmol, Amersham Biosciences; Piscataway, NJ) labeled PCR products of individual subtraction clones overnight at 65°C in a hybridization oven (Fisher Scientific; Pittsburgh, PA). After multiple washes in 1X, 0.5X and 0.2X SSC, membranes were air dried and radioactivity was analyzed and quantitated as described above .

Western Blot Analysis: TSP1 concentration was determined semi-quantitatively by Western Blot. HMVECs were treated with CGS-21680 or MRE0094. After 24 hrs treatment, supernates were collected. 20uL of protein supernates were fractionated on a 7.5% SDS–PAGE and transferred onto a nitrocellulose membrane (BioRad; Hercules, CA). After transfer, the nitrocellulose membrane was stained with 0.1% Ponceau (Sigma; St. Louis, MO) and scanned with a Microtek scanner. The net intensities were measured with Kodak Digital Science 1D, version 2.0.3. After removing the stain with distilled water, the nitrocellulose membrane was blocked for four hours at 4^{o}C in blocking solution (3% nonfat dry milk in 1x TTBS). The membrane was incubated at

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4^{o}C overnight on a rocker with blocking solution containing a 1:200 dilution of primary antibody (200ug/ml) for anti-angiogenic factor TSP1 (Santa Cruz; CA) and thereafter incubated with alkaline phosphatase labeled anti-goat secondary antibody (Santa Cruz; CA). The protein bands were visualized using the ECF kit (Amersham Biosciences; UK) and quantitated with Imagequant intensity software program. Ponceau staining was used as a normalization control.

Angiogenesis in vitro Assay: HMVEC were cultured with EBM-2MV media up to 80-90% confluence in a T75. HMVEC suspension was prepared by digesting the cell monolayers with trypsin and resuspending in culture medium. 50µl of matrigel (Chemicon; Temecula, CA) was loaded into each well. 200ul of cell suspension ($5x10^4$ cells/ml) along with A_{2A} agonist CGS-21680 or MRE0094 ranging from 10-5M to 10-9M was added to each well. Appropriate antagonist (ZM, enprofylline or DPCPX, each at 10uM) or the antibodies (anti-TSP1, anti-Collagen1 or anti-CD36, each at 1:50 dilution; Santa Cruz, CA) or exogenouse TSP1 protein at 10ug/ml were added. The angiogenesis assay plate was incubated for 16-18 hrs (37°C and 5% CO_2). Fluorophore, Calcein Am (Molecular Probes; Eugene, OR) was used to stain the tubes. Image acquisition of endothelial cell tube formation was achieved by MetaMorph software coupled with an automated imager. Sigma Scan Pro software was used to measured tube length and tube surface area; two parameters to quantitate the overall tube formation.

Statistical Analysis: Differences between groups were analyzed by means of one-way and two-way analyses of variances using SigmaStat (SPSS, Inc.; Chicago, IL). Data are presented as mean ± SEM. Differences with a p value of <0.05 were considered significant.

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RESULTS

Adenosine A_{2A} receptor agonist modulates gene expression in endothelial cells.

To characterize adenosine A_{2A} receptor activation effects on endothelial cells, we used a sensitive PCR-based subtraction approach to isolate genes expressed after 4h treatment with the selective A_{2A} agonist CGS-21680. Our approach combined an adaptation of the genomic representational difference analysis (RDA) method to cDNA analysis with a physical separation method (magnetic bead depletion). We isolated a total of 175 clones, 6 of which did not contain an insert, 34 did not match any known gene, 3 contained inserts that match B-actin but by southern blot they were not modulated, and the rest are summarized in Table II.

Adenosine A_{2A} receptor occupancy suppresses HMVEC expression of TSP1 mRNA.

Representational display analysis of mRNA from CGS-21680-treated and medium-treated human umbilical vein endothelial cells indicated that TSP1 message was modulated in the CGS-21680-treated cells. To confirm that TSP1 message was, in fact, downregulated by A_{2A} receptor ligation we determined TSP1 mRNA levels in medium and 1uM CGS-21680-treated human microvascular endothelial cells (HMVEC). Northern blot analysis and semi-quantitative RT-PCR demonstrated that CGS-21680 suppressed TSP1 message expression (Figure 1) corroborating the results of the representational display analysis. Representational display analysis further indicated that expression of a number of other genes might also be regulated by adenosine A_{2A} receptor occupancy (Table II) although the effects of adenosine A_{2A} receptor occupancy on expression of these gene products has not been confirmed.

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Adenosine A_{2A} receptor occupancy suppresses HMVEC secretion of TSP1 protein.

We next examined the effects of adenosine A_{2A} receptor ligation on expression of TSP1 protein by semi-quantitative western blot analysis. The selective adenosine A_{2A} receptor agonist CGS-21680 suppressed TSP1 secretion by HMVEC in a dose-dependent fashion (EC_{50} = 6.65nM, Figure 2). To confirm the identity of the adenosine receptor involved in suppression of TSP1 we determined the effect of selective adenosine receptor antagonists on the capacity of CGS-21680 to suppress TSP1 secretion by HMVECs. HMVECs express message for adenosine A_{2A} and A_{2B} receptors but not A_1 or A_3 receptors (Nguyen et al., 2003). The selective adenosine A_{2A} receptor antagonist ZM241385 (at both 10uM and 10nM concentrations), but not the A_{2B} or A_1 receptor antagonists enprofylline, MRS1706 or DPCPX, respectively, completely reversed the effect of CGS-21680 on TSP1 secretion (Figure 2). Studies of the effect of another A_{2A} receptor agonist, MRE0094, on TSP1 secretion were nearly identical to those obtained with CGS-21680; MRE0094 inhibited TSP1 secretion in a dose-dependent fashion (Figure 3, EC_{50} = 0.23μM). As with CGS-21680, ZM241385 but not enprofylline, MRS1706 or DPCPX reversed the effects of MRE0094 on TSP1 secretion (Figure 3). Thus, pharmacologic studies indicate that occupancy of adenosine A_{2A} receptors suppresses secretion of the anti-angiogenic protein TSP1.

Adenosine A_{2A} receptor occupancy enhances vascular tube formation *in vitro*.

To determine whether the suppression of TSP1 secretion by adenosine A_{2A} receptors was relevant to angiogenesis we determined the effect of adenosine A_{2A} receptor ligation on vascular tube formation by HMVECs cultured on matrigel. Both CGS-21680 and MRE0094 stimulated vascular tube formation in a dose-dependent fashion (EC_{50}= 0.1 μM, 185.4 ± 21.6% and 240.4 ± 67.9%, respectively, Figures 4-5). Interestingly, at a concentration at which CGS-21680 loses its selectivity for the A_{2A} receptor and stimulates the A_{2B} receptor, the effect on vascular tube

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formation is less marked (Figure 5A). MRE0094 stimulates vascular tube formation at all concentrations tested (Figure 5B). As with the effect of both CGS-21680 and MRE0094 on TSP1 secretion, ZM241385, but not enprofylline or DPCPX, completely abrogated the effect of both MRE0094 and CGS-21680 on vascular tube formation (Figures 4-5).

Antibody-mediated blockade of TSP1 and CD36, the cell surface receptor for TSP1, abrogate the effect of adenosine A_{2A} receptor occupancy on vascular tube formation.

The effects of adenosine A_{2A} receptor occupancy on TSP1 mRNA expression and protein secretion parallel those of A_{2A} receptor occupancy on vascular tube formation. To determine whether these two phenomena are related we determined the effect of anti-TSP1 antibody on the capacity of adenosine A_{2A} receptor occupancy to stimulate vascular tube formation. Anti-TSP1, but not anti-Collagen 1, antibodies increased vascular tube formation. As expected, in the presence of anti-TSP1 antibodies, the effects of CGS-21680 and MRE0094 on vascular tube formation were completely abrogated. As expected exogenous TSP1 inhibits vascular tube formation and, neither CGS21680 nor MRE0094 increase tube formation in the presence of exogenous TSP1 (Figure 6) TSP1 promotes its anti-angiogenic activity by binding to CD36 on the surface of HMVEC. Like antibodies to TSP1, antibodies to CD36 increased vascular tube formation and completely blocked the adenosine A_{2A} receptor-mediated promotion of vascular tube formation (Table III). These results are consistent with the hypothesis that adenosine A_{2A} receptor ligation promotes vascular tube formation (a measure of new vessel formation) by suppressing endogenous production of the anti-angiogenic matrix protein TSP1.

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DISCUSSION

The results reported here demonstrate that adenosine A_{2A} receptor occupancy promotes angiogenesis, at least in part, by downregulating the expression of TSP1, an anti-angiogenic matrix protein. Two different adenosine A_{2A} receptor agonists diminish TSP1 secretion in a dose-dependent manner and an antagonist of adenosine A_{2A}, but not A_1 or A_{2B} receptors, completely reverses this effect. Following A_{2A} receptor ligation, diminished TSP1 production is paralleled by diminished intracellular mRNA content. Similarly, these same adenosine A_{2A} receptor agonists increase vascular tube formation by cultured human microvascular endothelial cells and a selective A_{2A} receptor antagonist blocks enhanced vascular tube formation. Moreover, when the effect of endogenously-released TSP1 on vascular tube formation is blocked by specific antibodies for TSP1 and its receptor CD36, there is no adenosine A_{2A} receptor-mediated enhancement of angiogenesis in this model.

TSP1, a 420 kDa glycoprotein, is a naturally occurring inhibitor of angiogenesis which is synthesized and secreted by various cell types, including fibroblasts and endothelial cells in response to thrombin stimulation. It is a homotrimer in which each subunit possesses multiple structural domains which specify distinct biological functions through interaction with specific receptors on the effector cells. TSP1 is widely distributed in the extracellular matrix of numerous tissues and is degraded by both extracellular and intracellular routes. TSP1 influences cell adhesion, motility, growth and angiogenesis(Adams and Lawler, 2004; Ann Elzie and Murphy-Ullrich, 2004; Armstrong and Bornstein, 2003; Iruela-Arispe et al., 2004). The anti-angiogenic activity of TSP1 involves interaction with the microvascular endothelial cell receptor CD36, a transmembrane glycoprotein binding site for TSP1 on endothelial cells (Armstrong and Bornstein, 2003; Simantov and Silverstein, 2003). The effects of TSP1 on angiogenesis are

complex; TSP1 in the matrix also activates latent TGF-β (Guo et al., 1997) which stimulates granulation tissue (including blood vessel) formation in wounds. It is not clear whether the angiogenic or anti-angiogenic effect dominates at any given site although in the simple *in vitro* angiogenesis model studied the effect of reducing TSPI was to promote vascular tube formation.

Previous studies indicate that adenosine A_{2B} receptors may mediate enhanced angiogenesis (Afzal et al., 2003; Feoktistov et al., 2002; Feoktistov et al., 2003; Grant et al., 2001) although studies in cultured human endothelial cells and models of wound healing in A_{2A} receptor knockout mice are consistent with an A_{2A} receptor-mediated phenomenon (Montesinos et al., 2002; Nguyen et al., 2003). Human microvascular endothelial cells express mRNA for both adenosine receptor subtypes (Nguyen et al., 2003) and it is possible that both contribute to the angiogenic effects of exposure to CGS-21680 but not MRE0094. The evidence for a role of A_{2B} receptors in promoting angiogenesis is derived from studies performed with a human endothelial cell line (HMEC-1) or canine retinal endothelial cells whereas our studies have been performed with human microvascular endothelial cells and *in vivo* studies in mouse skin. The diverse origins of the cell types involved may explain the different effects of the two different adenosine A_2 receptors on angiogenesis reported to date.

As described above, both adenosine A_{2A} and A_{2B} receptors modulate endothelial cell function although the intracellular signaling mechanisms are unclear. Both A_{2A} and A_{2B} receptors are linked to $G_{\alpha S}$ signaling proteins in most cell types and this G protein is linked to activation of adenylate cyclase. In contrast, human microvascular endothelial cells do not express $G_{\alpha S}$ protein or message (Nguyen et al., 2003) but express G_{olf} which may also signal via adenylate cyclase. Moreover, without pre-treatment with TNF or IL-1 no cAMP response is detectable in adenosine A_{2A} receptor-stimulated cells (Nguyen et al., 2003). In a parallel fashion

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the adenosine A_{2A} receptor-stimulated increase in VEGF message is significantly greater in human microvascular endothelial cells pre-treated with TNF. Grant, et al (Grant et al., 2001) have reported that adenosine A_{2B} receptor occupancy promotes angiogenesis via stimulation of MAPKinase activity. The capacity of adenosine A_{2A} receptors to stimulate MAPKinase activity in human microvascular endothelial cells has not been tested and it is possible that the A_{2A} receptor signals by a mechanism shared with A_{2B} receptors.

The effects of both MRE0094 and CGS21680 on TSP1 secretion appear to be biphasic. Since HMVEC express neither A1 nor A3 receptors and the pharmacologic data are consistent with an A_{2A}R mediated effect it is difficult to associate the effects of these agents to another adenosine receptors. It is possible that A_{2A}R agonist sensitize the A_{2A}R to further stimulate even the prolonged inhibitions studied here. This phenomenon has not previously been reported. Alternatively there is a fifth type of adenosine receptor which is active at higher concentrations of CGS21680 that is responsible, in part, for the phenomenon observed here.

Our studies identify A_{2A} receptor-activation as a key switch for turning on angiogenesis, similar to Hepatocyte growth factor/scatter factor stimulation. Hepatocyte growth factor/scatter factor acts in parallel with A_{2A} receptor-stimulated phenomena: the ligand-receptor interaction stimulates endothelial cells to proliferate and migrate in vitro, induces blood vessel formation *in vivo* (Bussolino et al., 1992; Grant et al., 1993; Rosen and Goldberg, 1995), induces VEGF expression in human cancer cells, microvascular endothelial cells and monocytoid cells and diminishes TSP1 (Dong et al., 2001). TSP1 shut-off plays an important role in tumor suppression; ectopic expression of TSP1 markedly inhibits tumor formation through the suppression of angiogenesis (Zhang et al., 2003). Since ischemic tumors release large amounts of adenosine (Gao et al., 2001) there may be a role for adenosine in promoting tumor growth by

suppressing TSP1 production as well as the immune response to the tumor. Thus, the observation that adenosine A_{2A} receptors promote angiogenesis *in vitro* and *in vivo* suggests that specific adenosine A_{2A} receptor antagonists may be useful in the therapy of angiogenesis-dependent tumors.

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FOOTNOTES:

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FIGURE LEGENDS

Fig 1. CGS-21680 diminishes mRNA levels of thrombospondin 1 (TSP1) in human microvascular endothelial cells (HMVEC).

HMVEC were incubated with CGS-21680 (1μM) for 4hrs. in medium prior to cell lysis and harvest of total cellular RNA, as described. *A,* Representative RT-PCR of TSP1 mRNA expression in HMVEC treated with CGS-21680. *B,* Northern blot analysis of TSP1 message in HUVEC treated with CGS-21680.

Figure 2. Occupancy of adenosine A_{2A} receptors by CGS-21680 diminishes production of TSP1 in HMVEC.

HMVEC were treated with medium alone or varying concentrations of CGS-21680 alone or in the presence of the A_{2A} receptor antagonist (ZM241385, 10nM or 10μM), the A_{2B} receptor antagonist (Enprofylline 10μM or MRS1706 at 10nM) or the A_1 receptor antagonist (DPCPX, 10μM) overnight, and supernates were collected and proteins separated by SDS-PAGE prior to transfer to nitrocellulose membranes and immunoblotting, as described. Densitometric analysis of Western Blots was performed, as described and data are expressed as percentage inhibition. *A,* Representative Western blot for TSP1 protein. *B,* CGS-21680 diminishes TSP1 concentration in supernates in a dose-dependent fashion. Data are expressed as mean SEM percentage of inhibition (n=3) and there is a significant difference between the curves of dose-responses of cells treated with CGS-21680 alone and those treated with CGS-21680 plus ZM241385 ($p<0.001$, 2-Way ANOVA).

Figure 3. Occupancy of adenosine A_{2A} receptors by MRE-0094 diminishes production of TSP1 in HMVEC.

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HMVEC were treated with medium alone or varying concentrations of MRE0094 alone or in the presence of the A_{2A} receptor antagonist (ZM241385, 10μM or 10nM), the A_{2B} receptor antagonist (Enprofylline, 10μMor MRS1706 at 10nM) or the A_1 receptor antagonist (DPCPX, 10μM) overnight, and supernates were collected and proteins separated by SDS-PAGE prior to transfer to nitrocellulose membranes and immunoblotting, as described. Densitometric analysis of Western Blots was performed, as described and data are expressed as percentage inhibition. *A,* Representative Western blot for TSP1 protein. *B,* MRE-0094 diminishes TSP1 concentration in supernates in a dose-dependent fashion. Data are expressed as mean SEM percentage of inhibition (n=3) and there is a significant difference between the curves of dose-responses of cells treated with MRE-0094 alone and those treated with MRE-0094 plus ZM241385 ($p<0.01$, 2-Way ANOVA).

Figure 4. Fluorescence microscopy of *in vitro* vascular tube formation in untreated and CGS-21680 or MRE0094-treated HMVEC in the presence of selective antagonists.

HMVEC were cultured overnight on Matrigel and vascular tube formation was quantitated morphometrically following Calcein uptake and visualization by fluorescence microscopy, as described in Materials and Methods. Shown here are representative fields of vascular tube formation examined at a magnification of 2.5X.

Figure 5. CGS-21680 and MRE-0094 increase vascular tube formation *in vitro*.

HMVEC were cultured overnight on Matrigel in 96-well plates in the presence of either medium alone, CGS-21680 or MRE-0094, the A_{2A} receptor antagonist ZM241385, the A_{2B} antagonist Enprofylline or the A_1 antagonist DPCPX (all at 10μM), as described, before staining with Calcein and visualization under fluorescence microscopy. *A,* CGS-21680 increases vascular tube formation in a dose-dependent fashion. Data are expressed as the mean % control of vessel area

(SEM). CGS-21680 enhances vascular tube formation and this effect is abrogated in the presence of ZM241385 ($p<0.001$, 2-way ANOVA, n=3, 5, 3, 4 for CGS-21680, ZM241385, Enprofylline and DPCPX treated cells, respectively). *B,* MRE0094 increases vascular tube formation in a dose-dependent fashion. Data are expressed as the mean % control of vessel area (SEM). MRE0094 enhances vascular tube formation and this effect is abrogated in the presence of ZM241385 ($p<0.001$, 2-way ANOVA, n=6, 3, 3, 4 for MRE0094, ZM241385, Enprofylline and DPCPX treated cells, respectively).

Figure 6. Anti-TSP1 antibodies abrogate the effect of A_{2A} receptor agonists CGS-21680 or MRE94 on *in vitro* vascular tube formation.

A, HMVEC were incubated on Matrigel overnight in the presence of CGS-21680 alone, goat anti-TSP1 IgG, goat anti-Collagen I IgG or exogenous TSP1 protein. Data are expressed as mean % control (SEM), as described. The effect of CGS21680 on tube formation was statistically significant ($p=0.005$, n=3, ANOVA). Anti-TSP1 IgG alone significantly increased vascular tube formation to 184 +/- 33% of control ($p=0.005$, n=7, 2 way ANOVA) and there was no further effect of CGS21680 in the presence of anti-TSP1. In the presence of anti-Collagen 1 CGS21680 significantly increased tube formation ($p<0.001$, n=3, 2 way ANOVA). Exogenous TSP1 significantly decreased tube formation and CGS21680 did not increase tube formation in presence of TSP1 ($p<0.001$, n=3, 2 way ANOVA). *B,* HMVEC were incubated on Matrigel overnight in the presence of MRE-0094 alone, goat anti-TSP1 IgG, goat anti-Collagen I IgG or exogenous TSP1. The effect of MRE0094 on tube formation was statistically significant ($p<0.001$, n=4, ANOVA). Anti-TSP1 IgG alone significantly increased vascular tube formation to 162.9 +/- 12.2 % of control (p=NS, n=3, 2 way ANOVA) and there was no further effect of MRE0094 in the presence of anti-TSP1. In the presence of anti-Collagen 1 MRE0094

significantly increased tube formation ($p=0.018$, $n=3$, 2 way ANOVA). Exogenous TSP1 significantly decreased tube formation and MRE0094 did not increase tube formation in presence of TSP1 ($p<0.001$, $n=3$ 2 way ANOVA).

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Tables:

Table I. Human adenosine receptor binding affinities

	Adenosine receptor binding affinities *Ki (nM)*			
	A1	***A_{2A}***	***A_{2B}***	***A3***
CGS21680[1]	*290*	*27*	*88800 (EC50)*	*67*
MRE0094[2]	*>10000 (IC50)*	*490 ± 50 (IC50)*	*> 10000 (IC 50)*	*----*
ZM241385[1]	*540*	*1.4*	*31*	*270*
DPCPX[1]	*3.9*	*130*	*50*	*4000*
Enprofylline[3]	*156000*	*32000*	*7000*	*65000*
MRS 1706[4]	*157*	*112*	*1.39*	*230*

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Table II. Genes modulated by A_{2A} receptor agonist CGS-21680 in HUVEC.

HUVEC were incubated for 4 hours in the presence or absence of 1μM selective adenosine A_{2A} receptor agonist, CGS-21680. The subtraction library was generated using the cDNA representational difference analysis as described in materials and methods. Products larger than 164 bp indicate an insert had been cloned. PCR products of inserts from individual clones were identified were analyzed using Sequencher™ and compared to public databases by using BLAST. Column 1 indicates the number of hits for the protein mentioned in column 2.

Number of clones	Homology (>99%)
5	Thrombospondin 1
5	Laminin beta 1
2	Catenin alpha
1	Catenin isoform 1A
3	Integrin-linked kinase
2	Karyopherin beta 1
4	Transmembrane protein with EGF and follistatin-like domains
2	Gap junction protein, connexin 43
1	H-cadherin 13
1	Human type XII collagen alpha 1
5	Mouse type XVIII collagen alpha 1

2	MUC-18, cell surface glycoprotein
2	Alpha spectrin
1	Type II receptor for morphogenic proteins
1	Human C/EBP gamma for IL-4 promoter
1	2,3-biphosphoglycerate
1	Similar to basonuclin
1	p40
1	ZNF127-xp zinc finger protein
1	Mitogen responsive phosphoprotein (DOC-2)
1	Protein phosphatase 2A, alpha subunit
1	Protein tyrosine phosphatase alpha
2	Ca^{++} ATPase
3	Proteosome ATPase
1	ATP synthase, alpha subunit
2	Ras GTPase activating protein SynGAP-C
1	UMP Synthase
2	UMP Synthase and mitochondrial elongation factor
2	Elongation factor 2
1	Asparaginyl-tRNA synthethase
8	Glycyl-tRNA synthethase
2	Microtubule associated protein 1B
2	Splice factor SRp30c
1	CGG trinucleotide repeat binding protein

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1	Tim17 preprotein translocase
1	Nuclear distribution protein C
2	Ribosomal protein P0 and glycyl-tRNA synthethase
6	Ribosomal protein P0
1	Acidic ribosomal protein P0, P1 and P2
3	Mitochondrial genome
3	EXT1 tumor suppressor gene
1	Tumor suppressor p33 ING1 homolog
1	Excision repair gene
1	Excision repair gene and KIAA0251
3	CGI-74 orCGI-59
1	IDN3
1	PAC 30p20
1	PAC 12q24
1	CAD
9	Human clone 24901
12	KIAA0251
1	KIAA0676 (BUB2-like protein)
1	KIAA0333
1	KIAA0230 (similar to peroxidasin)
2	KIAA0091 (site-1 protease)
1	KIAA1013
1	KIAA0107

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1	KIAA0478 (zinc finger domain)
1	KIAA0225
1	KIAA1603

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Table III. Anti-CD36 antibodies abrogate the effect of A_{2A} receptor agonists CGS-21680 or MRE94 on *in vitro* vascular tube formation.

HMVEC were incubated on Matrigel overnight in the presence of CGS-21680 or MRE-0094 alone or in the presence of rabbit anti-CD36 IgG (n= 3). Data are expressed as mean % control (SEM), as described.

	Media	Anti-CD36
Control	100 ± 0	176 ± 0
CGS-21680 1μM	198 ± 33	180 ± 9
MRE0094 1μM	208 ± 42	185 ± 48

Fig 1

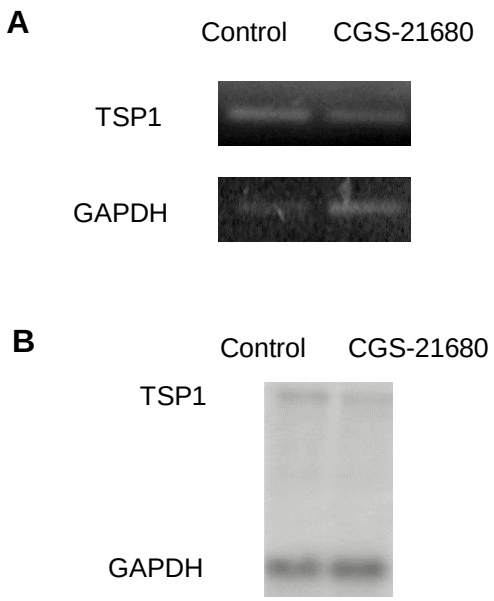

Fig 2

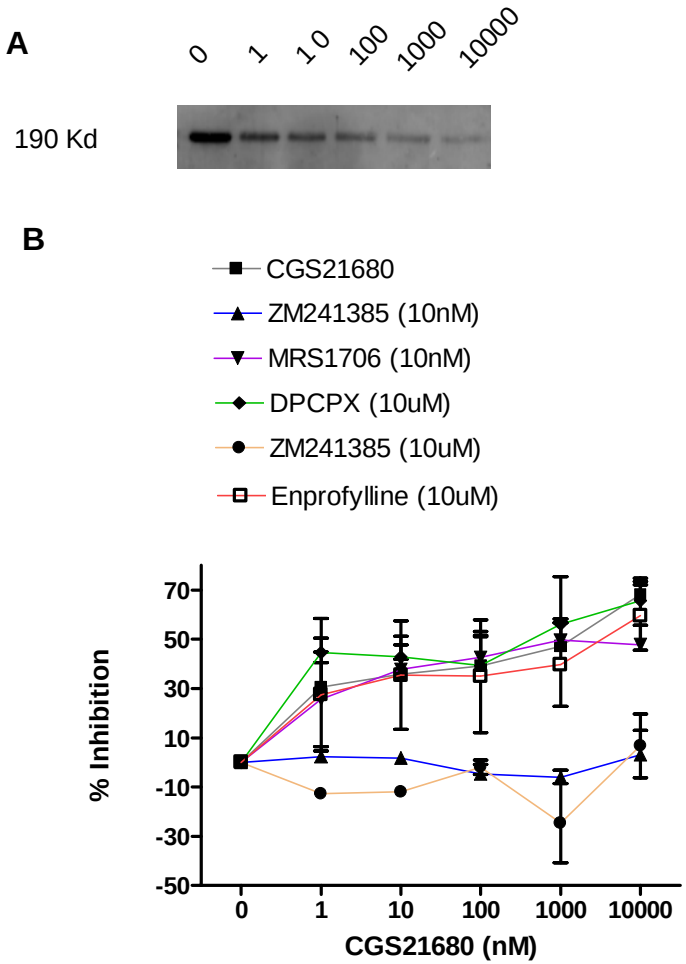

Fig 3

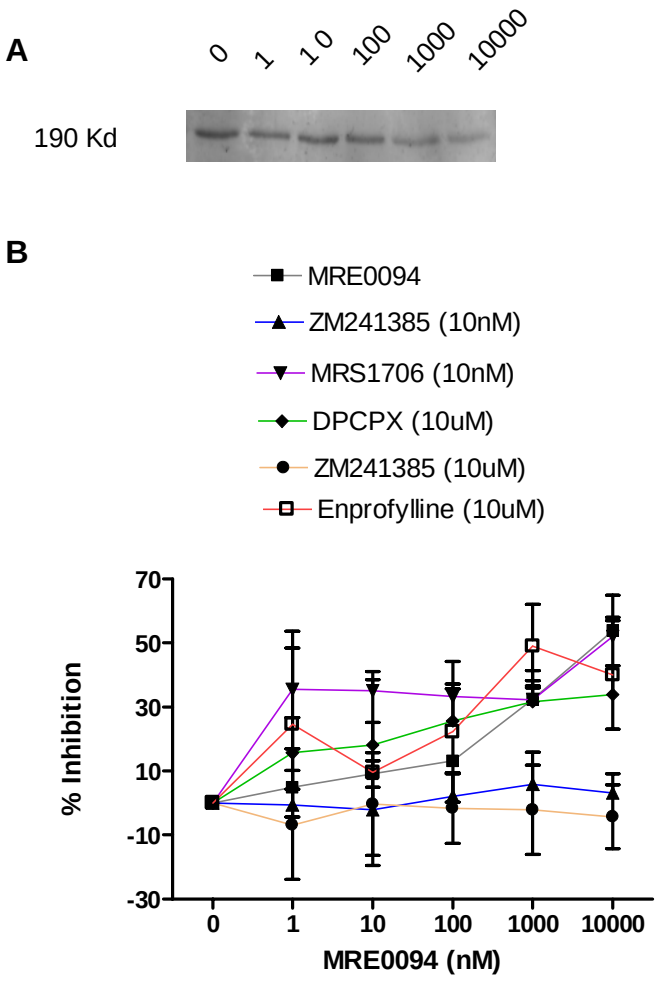

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Fig 4

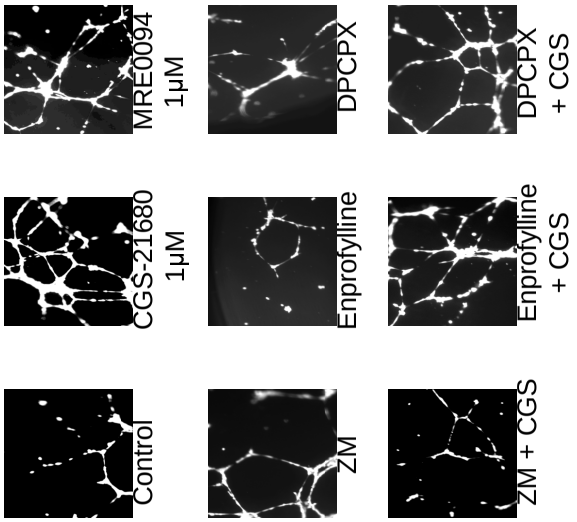

Fig 5

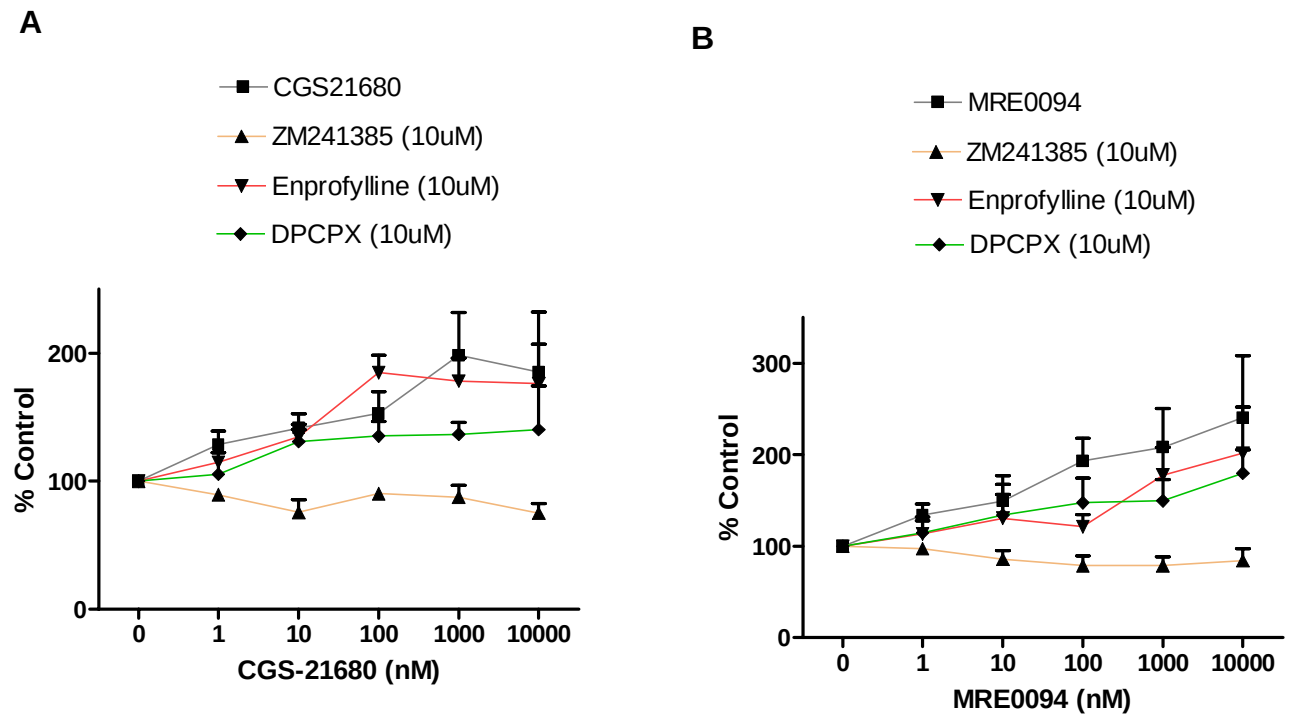

Fig 6