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Title:

A novel high-throughput screening system identifies a small molecule repressive for matrix metalloproteinase-9 expression

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Running title**:** **HTS system for agents regulating gene expression**

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Abbreviations:

ActD: Actinomycin D; AP-1: activator protein-1; FRT: Flp recombination target; HTS: high-throughput screening; JNK: Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MMP: matrix metalloproteinase; MPBD: 5-methyl-2-(4-methylphenyl)-1H-benzimidazol; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB: nuclear factor κB; PBD, 2-(4-methylphenyl)-1H-benzimidazole; PMA: phorbol myristate acetate; RANKL: receptor activator of nuclear factor-κB ligand; TRE: TPA responsive element.

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Abstract

Aberrant gene expression is one of the driving forces for cancer progression, and is considered an ideal target for chemical intervention. While emerging bioluminescence reporter systems allow high-throughput searches for small molecules regulatory for gene expression, frequent silencing of reporter genes by epigenetic mechanisms hinders wide application of this drug discovery strategy. Here we report a novel system that directs the integration of a promoter-reporter construct to an open chromosomal location by Flp-mediated homologous recombination thereby overcoming reporter-gene silencing. Employing this system, we have screened more than 8000 compounds in the DIVERSet chemical library for repressors of a MMP-9 promoter, and identified 5-methyl-2-(4-methylphenyl)-1H-benzimidazol (MPBD) inhibitory for MMP-9 gene expression. Consistent with this effect, MPBD inhibits MMP-9-dependent invasion of UMSCC-1 oral cancer cells, pre-osteoclast migration and RANKL-induced osteoclast activity over concentration ranges that repressed MMP-9 expression. Mechanistic studies indicated that MPBD antagonizes AP-1 function by inhibiting its *trans*-activation activity. We conclude that the Flp-mediated homologous recombination system to direct reporter integration into open chromatin regions represents a novel strategy allowing for the development of high-throughput systems screening for lead compounds targeting aberrant gene expression in cancer.

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Introduction

Aberrant gene expression is a hallmark of cancer. Expression of genes such as matrix metalloproteinase-9 (MMP-9, 92-kDa type IV collagenase / gelatinase B) (Van den Steen et al., 2002) that are essential for normal cellular homeostasis is often altered, resulting in growth and cancer progression. Therefore, targeting the expression of these genes with small molecules has been considered an attractive approach to prevent and/or eliminate cancer (Pandolfi, 2001). Indeed, in this post-genomic era, since many of the genes that are implicated in the genesis and progression of cancer have been identified, large-scale searches for lead compounds that serve as prototypes for therapeutic agents rectifying aberration of gene expression in cancer cells are imperative.

Due to their simplicity and sensitivity, reporter gene systems are often used for developing high-throughput assays screening for small-molecule gene regulators. Typically, a reporter (e.g. firefly luciferase) fused to a natural or synthetic promoter and stably-integrated into the genome of a cell line allows for high throughput, cost-efficient screening amenable to robotic manipulations (Shoemaker et al., 2002). An integrated or transgenic reporter has obvious advantage over a transiently-expressed reporter given that the latter has to be introduced into cells for every assay and transfection efficiency varies. Indeed, several groups have successfully employed this technology to identify compounds regulatory for the activities of transcription factors such as CEPBα (Shoemaker et al., 2002), p53 (Wang et al., 2006) or HIF-1 (Rapisarda et al., 2002) and their respective downstream targets. Notwithstanding these advances, such systems are nevertheless hindered by transgene position effects, a common phenomenon whereby the activity of reporter genes integrated into non-permissive chromosomal locations is either repressed or unstable due to a closed chromatin structure (Wakimoto, 1998; Yan and Boyd, 2006). Thus, reporters are silenced or variegated in their expression making them

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unsuitable for compound screening. Indeed, this is a real obstacle given that non-permissive chromosomal locations are widely distributed throughout the human genome (Barski et al., 2007) and that conventional DNA delivery approaches confer random, rather than site-specific, integration of transgenes.

Recently, we have developed a system that allows reporter genes to integrate at specified chromosomal locations tagged with a FRT sequence through Flp-mediated homologous recombination (Yan et al., 2004). This advance prompted us to ask whether this system could be exploited to introduce reporter genes into a specific chromosomal location where chromatin structure is open, and whether the derived cells are of utility for high-throughput compound screening. To address this, we have introduced a firefly luciferase reporter under the control of a 2.2-kb MMP-9 promoter into a HT1080 clone in which the FRT recombination fragment is located within an open chromatin region (F55 site) (Yan and Boyd, 2006), generating cells bearing an integrated MMP-9 promoter-driven reporter for high-throughput screening (HTS). We chose MMP-9 (matrix metalloproteinase-9, 92-kDa type IV collagenase/gelatinase B) as a test gene, given its role in promoting multiple steps across the tumor progression spectrum including angiogenesis, invasion/metastasis, and osteolysis (Van den Steen et al., 2002; Hjertner et al., 2005). MMP-9 is over-expressed in many cancers, and contributes to cancer progression in part by degrading extracellular matrix and activating pro-angiogenic growth factors such as TGF-β. Regulation of MMP-9 protein levels has been largely ascribed to transcriptional activation of the gene through binding sites for AP-1 and NFκB transcription factors that are localized in the 2.2-kb promoter region (Yan and Boyd, 2007), and therefore aberrant MMP-9 expression could be an ideal target for cancer therapy. Indeed, small molecules that inhibit the enzymatic activity of MMP-9 have been shown preclinical efficacy in the inhibition of growth and progression of various

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cancers in experimental models. However, these agents targeted many other metalloproteinases and thus caused unexpected side effects in clinical trials (Coussens et al., 2002), mandating a need to search for small molecules that directly target MMP-9 expression.

Employing this novel system, we have identified a small molecule that inhibits MMP-9 expression without affecting the expression of 2 related metalloproteinases. Importantly, this compound counters various MMP-9-dependent biological events including cancer cell invasion, osteoclast migration and activity, and thus represents a new class of MMP-9 antagonists that could be of therapeutic benefit. These results collectively demonstrate that this Flp-mediated homologous recombination system represents a useful technology for developing HTS assays to screen compound libraries for regulators of gene expression for cancer therapy.

Materials & Methods

Cell culture and transfections

HT1080 and UMSCC-1 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and antibiotics. RAW264.7 cells were cultured in DMEM-F12 medium. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Flp-mediated integration of the MMP-9 promoter

This was performed essentially as described previously (Yan et al., 2004). Briefly, 1 μg pCM/MMP-9/Luc2.2k, a FRT-harboring plasmid containing a luciferase gene driven by a 2.2-kb MMP-9 promoter (Yan et al., 2004), was co-transfected with 9 μg Flp-expressing pOG44 plasmid into HT1080/F55 cells

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(Yan and Boyd, 2006) in a 100 mm plate followed by selection of the transfected cells with 200 μg/ml Hygromycin B (Calbiochem, San Diego, CA). The obtained resistant clones were lysed for luciferase activity assays, while a random clone designated as HT/MMP9/F55 was expanded for HTS assays.

High-throughput compound screening

HT/MMP9/F55 cells were plated in 96-well plates at a density of 1.5×10^4 /well. On the following day, 5 μM compounds (final concentration) from the DIVERSet chemical library (ChemBridge, San Diego, CA) or a corresponding amount of DMSO was added and cells treated overnight (18 h). After washes with PBS, the cells were lysed in 100 μl Passive Lysis Buffer (Promega, Madison, WI), and 20 μl cell lysate assayed for luciferase activity as described previously (Yan et al., 2004).

Real-time RT-PCR and gelatin zymography

Total RNA was prepared, reverse transcribed and subjected to real-time PCR assays essentially as described previously (Yan and Boyd, 2006). The primers sequences used for amplifying MMP-9, MMP-2 and β-actin cDNA were described in our previous report (Yan et al., 2001). MMP-1 primer sequences were 5'-GGA GAT CAT CGG GAC AAC T-3' and 5'-GGG TAT CCG TGT AGC ACA TTC-3'. PCR specificity was confirmed by the identification of a single amplified product in an agarose gel. Zymography was carried out as described previously (Yan et al., 2001), using aliquots of conditioned medium corrected for any differences in cell number.

AP-1 reporter assays, c-Jun *trans* -activation assays and Western blotting

To determine AP-1 activity, HT1080 cells were plated in 96-well plates and transfected with 0.1 μg pAP-1-luc containing 7 tandem repeats of AP-1 consensus binding sites (TRE, see Fig. 6B)

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(Stratagene, La Jolla, CA). 24 h later, MPBD was added and treated the cells overnight (16 h) before the cells were lysed for luciferase activity assays as described (Yan et al., 2004). To determine the *trans*-activation activity of c-Jun, the PathDetect system (Stratagene, La Jolla, CA) was used according to the manufacturer's recommendations (Shin et al., 2002). Briefly, HT1080 cells were transfected with pFR-luc (expressing a Gal4-driven luciferase reporter, see Fig. 6C), pFAc-Jun (expressing a Gal4-c-Jun fusion protein) or pFCdbd empty vector in 96-well plates. 24 h post- transfections, compound MPBD was added and treated the cells for an additional 16 h. The luciferase activity was determined as described (Yan et al., 2004). Western blotting was performed as described previously (Yan et al., 2001). The antibodies against phosphorylated c-Jun (Ser73) (cat# 9164) was purchased from Cell Signaling Technology (Danvers, MA).

Invasion and Motility assays

For invasion assays, UMSCC-1 were detached with 2 mM EDTA, suspended in serum-free medium containing 1% BSA, and 5×10^4 cells plated into a Matrigel-coated Transwell. Invaded cells were stained and quantitated using a Cell Invasion Assay Kit (Chemicon, Temecula, CA) 20 h later following the manufacturer's instruction. For cancer cell-mobilized migration assays, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 72 h, detached and suspended as above. Cells (5×10^4) were plated into a Transwell, and the latter inserted into a 24-well plate containing 1×10^5/well UMSCC-1 cells. Migrated RAW264.7 cells were stained and enumerated 40 h later.

TRAP staining and osteolcastic activity assays

TRAP staining to determine osteoclastogenesis were performed using a Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, IL) according to the manufacturer's recommendations. Briefly, RAW264.7 cells

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were stimulated with 100 ng/ml RANKL for 4 days, and then fixed in Citrate/Acetone solution for 30 seconds. After rinsing and drying, the cells were incubated with a mixture containing Acetone solution, Napthol AS-BI phosphoric acid, Tartrate solution and Fast Garnet GBC salt at 37°C for 1 h, and observed under a microscope. For osteoclastic activity assays, RANKL-stimulated cells were detached, and suspended in RANKL-containing medium with or without 20 μM MPBD, and plated on 16-well coated quartz slides (BioCoat Osteologic Bone Cell Culture System, BD Biosciences, Bedford, MA). After 3 days, the cells were washed with PBS followed by addition of Bleach for 3 min. After extensive washes, the slides were dried and images were captured using a light microscope (40 ×). Quantitation of the resorbed area of the bone was with Image J software.

Results

Integration of a MMP-9 promoter into an open chromosomal location through homologous recombination

The first and critical step towards developing a reporter gene-based system for high-throughput compound screening is to establish cells that stably express the reporter gene under the control of a promoter of interest. Due to transgene position effects, reporter genes are often silenced, necessitating that the reporter gene is directed to an open chromosomal location. We have previously characterized the F55 site as a "permissive" chromosomal location that is defined by histone H3 acetylation and H3 K4 methylation and not subject to transgene silencing over extended periods (Yan and Boyd, 2006). We thus employed the Flp-mediated homologous recombination system as described in our previous report (Yan et al., 2004) to direct the integration of a luciferase cDNA driven by a 2.2-kb MMP-9 promoter at this chromosomal location. Toward this end, we co-transfected the endogenous MMP-9-expressing HT1080 cells harboring the F55 site (Yan and Boyd, 2006) with a Flp-expressing plasmid

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and the promoter-luciferase construct that also carries a FRT fragment (Fig. 1A). After selection with Hygromycin B for two weeks (Yan et al., 2004), we obtained several resistant clones that ubiquitously expressed high levels of luciferase (data not shown), consistent with our previous report that the F55 location is permissive for transgene expression (Yan and Boyd, 2006). Since these isogenic clones expressed comparable levels of luciferase, we randomly expanded one clone (HT/MMP9/F55) and determined whether these cells respond to a known MMP-9 stimulus (PMA) (Yan et al., 2004). The cells were plated into a 96-well plate at different densities (1.0×10^4, 1.5×10^4, 2.0×10^4, 2.5×10^4, and 3.0×10^4/well), and subjected to treatments with varying amounts of PMA. As expected, PMA increased the cellular luciferase activity in a dose-dependent manner over a wide range of cell density (Fig. 1B), suggesting that the activity of the transgenic MMP-9 promoter mirrors its endogenous counterpart, and thus alterations in the expression level of the reporter gene likely reflect altered transcription of the endogenous MMP-9 gene. We chose a plating density of 1.5×10^4/well for the subsequent HTS studies since the cells plated at this density grew as a nearly confluent monolayer and well responded to the chemical treatment (Fig 1B).

High-throughput screening for compounds inhibiting MMP-9 promoter activity

We then employed these cells for high-throughput screening for compounds repressive for MMP-9 expression. Toward this end, we treated HT/MMP9/F55 cells cultured in 96-well plates with 5 μM of individual compounds from the DIVERSet library (comprised of > 8,000 compounds), and then measured the luciferase activity as an index of promoter activity. To ensure that the assay conditions have been optimized, we first treated two plates of cells with 1 μM of Actinomycin D (positive control), a general transcription inhibitor (Sobell, 1985) which also repressed the transgenic MMP-9 promoter (Fig 1C), and calculated assay variability within and between plates. The intra-plate

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variability (CV) ranged from 10.9% to 12.1%, while the plate-to-plate variation was negligible (Fig. 1D). In addition, the value of Z′-factor (Zhang et al., 1999) equaled to 0.64 (Fig. 1D), indicating that the assay system could be used for high-throughput screening for MMP-9 repressing agents. Moreover, to further ensure the reproducibility of the screening data, we performed a small scale screening with 240 compounds from the library, and generated a Q-Q plot (Huang et al., 2004) based on the effects of these compounds on the transgenic promoter in two independent treatments. The quantile distributions derived from the duplicate assays (scatter squares) only slightly deviated from the quantiles of a normal distribution (straight line) (Fig. 1E), indicating a high reproducibility between independent experiments (Huang et al., 2004).

We thus screen the entire library (8471 compounds) for small molecules repressive for transgenic promoter activity. We excluded those compounds that reduced luminescence signal to the background level since they most likely interfered with the luciferase-enzyme reaction or were severely toxic to the cells. While the majority of compounds (94.8%) had no, or modest effects (relative luciferase activity greater than 0.5 but smaller than 2 - $\log_2$ scale), 5.2% either increased (405 compounds or 4.8%) or reduced (35 or 0.4%) the luciferase activity by 1 fold (relative luciferase activity greater than 2 or smaller than 0.5 - $\log_2$ scale) (Fig. 2A). Of the latter group, 4 compounds (7561157, 7642828, 7661218, and 5691508) yielded a greater than 75% reduction in luminescence signal (Fig. 2A) and were therefore pursued for further investigation. We confirmed the effects of these compounds in an independent experiment showing that luciferase activity was dramatically reduced in a dose-dependent manner (Fig. 2B). Compound 7561157 and 7642828 resulted in 90% reduction in the luciferase activity at a concentration as low as 2.5 μM (Fig. 2B). Cytotoxicity assays (Fig. 2C) ruled out the possibility that repressed reporter activity was merely a consequence of cell death.

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A benzimidazole derivative specifically represses MMP-9 expression

Next, we validated the effect of these 4 small molecules on endogenous MMP-9 expression. We first treated HT1080 cells with these compounds, and measured MMP-9 protein levels with zymography. Of the 4 compounds, 7561157, a benzimidazole derivative (5-methyl-2-(4-methylphenyl)-1H-benzimidazole) referred to as MPBD hereafter (see Fig. 7A for the chemical structure), dose-dependently inhibited PMA-induced elevation of MMP-9 levels (Fig. 3A), but had minimal effect on the basal MMP-9 level and the level of MMP-2 a constitutively-expressed gelatinase functionally related to MMP-9 but differing in that it is unresponsive to PMA (Sternlicht and Werb, 2001) (Fig. 3A). This effect was most likely attributable to the repression of MMP-9 transcription evident by the reduction of MMP-9 mRNA levels as measured by real-time RT-PCR (Fig. 3B), and consistent with the result from a reporter assay showing that MPBD inhibited PMA-induced *trans*-activation of the integrated MMP-9 promoter (data not shown). This observation is notable since PMA stimulates the MAPK signaling pathway a convergence node for divergent growth factors (Van den Steen et al., 2002). Indeed, 20 μM MPBD reduced MMP-9 induction by 80% (Fig. 3B). The lack of effect of MPBD on basal MMP-9 mRNA levels may reflect the prolonged half life of the MMP-9 transcript (~19 h) (Sehgal and Thompson, 1999) coupled with a low level of transcription under basal conditions. Interestingly, MPBD did not inhibit MMP-2 expression (Fig. 3C), while it only slightly repressed PMA-induced MMP-1 expression ($p>0.05$, Fig. 3D), although the promoter of the latter gene bears strong similarity to that of MMP-9 (Yan and Boyd, 2007).

MPBD inhibits cancer cell invasion and blocks pre-osteoclast migration and osteoclastic activity

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We then queried the ability of MPBD to interfere with 2 known biological functions of MMP-9 in cancer (a) invasiveness of cancer cells and (b) cancer-driven osteoclast activity. Regarding the former, we previously reported that the *in vitro* invasiveness of UMSCC-1 oral cancer cells, which do not express MMP-2, is partly dependent on MMP-9 expression (Juarez et al., 1993). Like HT1080 cells, MPBD also inhibited PMA-induced MMP-9 expression in this oral cancer line as evident with zymography (Fig. 4A). We thus treated UMSCC-1 cells with 20 μM MPBD for 24 h, stimulated them with PMA, and determined cell invasion using Transwell chambers coated with Matrigel. Consistent with its inhibition of MMP-9 expression (Fig. 4A), MPBD reduced the number of cells invading through the reconstituted basement membrane over an identical concentration range (Fig. 4B). Quantitation with a cell staining method (see Material and Methods) indicated that MPBD inhibited the invasiveness of UMSCC-1 cells by more than 70% (Fig. 4B).

As mentioned above, MMP-9 also contributes to bone resorption with some osteolytic malignancies including breast, multiple myeloma, oral cancers (Ash et al., 2000; Van Valckenborgh et al., 2004; Guise et al., 2005). For cancers metastatic to the bone, osteoclasts and their precursors (macrophage lineage) rather than cancer cells express MMP-9 in response to cytokines including receptor activator of nuclear factor-κB ligand (RANKL), resulting in bone lysis, cancer cell colonization/growth as well as further recruitment of pre-osteoclasts to the remodeling bone (Guise et al., 2005). Since MPBD inhibited MMP-9 expression in cancer cells, we asked whether it could also blunt MMP-9 expression in pre/osteoclasts, their recruitment/migration induced by cancer cells, and subsequent osteoclastic activity. First, we treated RAW264.7 osteoclast precursors with RANKL for 3 days and assayed for MMP-9 levels by zymography. As reported previously (Dong et al., 2005), RAW264.7 cells expressed low MMP-9 levels (no MMP-2 expression), whereas RANKL stimulation yielded a substantial amount

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of MMP-9 as detected by zymography (Fig. 5A). Treatment with MPBD, however, dramatically inhibited RANKL-induced MMP-9 expression (Fig. 5A) while 20 μM of this compound returned the MMP-9 amount to the basal level. Thus, this small molecule inhibited MMP-9 expression in osteoclast precursors as well. MPBD did not prevent RANKL-induced osteoclastogenesis (Fig. 5B), arguing against the possibility that the inhibition of MMP-9 expression was due to toxicity.

Since pre-osteoclast migration is MMP-9-dependent (Dong et al., 2005), we stimulated the RAW 264.7 osteoclast precursors with RANKL and determined the effect of MPBD on their migration across a Transwell filter induced by conditioned media from UMSCC-oral cancer cells. Oral cancers are known for the ability to promote osteolysis (Ash et al., 2000). As expected, RANKL increased cell migration across the membrane by 100% (Fig. 5C). Importantly, MPBD inhibited RANKL-induced migration (Fig. 5C), while 2-(4-methylphenyl)-1H-benzimidazole (PBD), a MPBD analog that lacks the 5-methyl group (Fig. 7A) and that failed to inhibit RANKL-induced MMP-9 expression (see lane 5, Fig. 7D), had no effect on migration of stimulated cells (Fig. 5C). These results suggest that MPBD effectively counters MMP-9-dependent pre-osteoclast migration (Dong et al., 2005).

Since MMP-9 secreted by osteoclasts contributes to bone destruction by osteolytic cancers (Guise et al., 2005), we also asked whether the repression of MMP-9 expression by MPBD counters osteoclastic activity. We thus pre-treated RAW264.7 cells with RANKL, plated them on a slide coated with a synthetic bone biomaterial mimicking bone tissue and measured osteoclastic activity. As expected, RANKL-stimulated RAW264.7 cells exhibited a marked osteoclastic activity as evident with the large “resorbed” areas as shown as clear, coating-free surfaces (Fig. 5D). In contrast, upon treatment of the osteoclasts with 20 μM MPBD, a concentration which reduces MMP-9 expression to basal level, the

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activity of these cells in this assay was almost completely abrogated with only small areas of "resorption" now evident (Fig. 5D, arrows). Again, the efficacy of MPBD in strongly antagonizing osteoclast activity was not secondary to toxicity as indicated by the presence of TRAP-positive multinucleated cells after MPBD treatment (Fig. 5E, arrows). These biological data provide further support for the view that MPBD is a novel repressor of MMP-9 expression.

MPBD reduces MMP-9 promoter activity by antagonizing AP-1 function

What is the mechanism underlying the repression of the MMP-9 promoter? The human MMP-9 promoter contains a proximal AP-1 binding site (-73) and a NF-κB motif (-600) (Yan and Boyd, 2007) crucial for regulating MMP-9 expression in response to diverse stimuli including PMA (Yan et al., 2004) and RANKL (Sundaram et al., 2007). We employed a set of HT1080 clones that harbor a single-copy wild-type MMP-9 promoter or mutants in the AP-1 or the NF-κB-binding-site in an identical chromosomal location (the F8 site) (Yan et al., 2004), and determined whether MPBD affected the activities of these transgenic promoters. The advantage of this system is that the flanking sequence is identical thus excluding the confounding influence of different neighboring sequences on reporter activity (Yan et al., 2004). As expected, this compound inhibited the wild-type MMP-9 promoter (Fig. 6A). In contrast, point mutation at the proximal AP-1 binding site (mTRE), but not the NF-κB binding site (mkB), compromised (although not completely abrogated) the inhibitory effect of MPBD on the MMP-9 promoter (Fig. 6A), consistent with the notion that the compound represses MMP-9 promoter activity in part via antagonizing AP-1 function at the proximal binding site. Indeed, MPBD dose-dependently repressed the activity of a transiently-expressed AP-1 reporter (pAP-1-luc, Fig. 6B) containing 7 tandem repeats of the AP-1 consensus binding site (Fig. 6B), confirming that AP-1 is one of the molecular targets of this small molecule. Nevertheless, it is worth emphasizing that since the

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repressive effect of MPBD was not completely blocked by the AP-1 mutation (Fig. 6A), it is likely that the inhibition of MMP-9 expression by this compound was mediated through multiple *cis* elements including AP-1.

One interesting question emerged was how MBPD antagonized the AP-1 function. Such an effect could be achieved by either inhibiting the AP-1 DNA-binding activity or repressing its *trans*-activation activity. Interestingly, while electrophoresis motility shift assays showed no evidence that MPBD altered the DNA-bound AP-1 protein level (data not shown), this small molecule significantly inhibited AP-1 *trans*-activation activity (Fig. 6C). Thus, while a transiently-expressed luciferase reporter driven by Gal4-binding *cis*-elements (pFR-luc, Fig. 6C) was strongly *trans*-activated by a c-Jun protein recruited to the promoter via a fused Gal4 DNA-binding domain (pFA c-Jun, Fig. 6C) (Shin et al., 2002), MPBD dramatically repressed the c-Jun *trans*-activation activity in a dose-dependent manner (Fig. 6C). This repressive effect was unlikely due to an inhibition of the general transcription machinery since the small molecule did not modulate expression of the basal reporter activity driven by the empty vector (Fig. 6C, see pFc-dbd). Therefore, MPBD inhibits MMP-9 expression partly through repressing AP-1 *trans*-activation activity.

Since phosphorylation of c-Jun by the Jun N-terminal kinase (JNK) contributes to AP-1 *trans*-activation activity (Bogoyevitch and Kobe, 2006), it might be that MPBD repressed AP-1 activity through inhibiting phosphorylation of the AP-1 family member. However, under the same conditions where AP-1 activity was repressed (Fig 6B and 6C), MPBD affected neither the phosphorylation level of the endogenous c-Jun (Fig 6D) nor that of the transfected Gal4-c-Jun fusion protein (Fig 6E), while a JNK inhibitor SP600125 (Shin et al., 2002) clearly inhibited the c-Jun phosphorylation (Fig 6E).

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These results suggest that the c-Jun/JNK pathway might not be the target of this novel AP-1 antagonist. Similarly, MPBD did not affect the activation of the MAPK pathway (data not shown), indicating that this compound may not target upstream cell signaling as well.

Analogs to MPBD inhibit MMP-9 expression

Since we have identified MPBD as a novel small molecule inhibiting MMP-9 expression, we asked whether it could serve as a lead compound for drug discovery. We thus queried the ChemBridge compound inventory (http://www.hit2lead.com/) for MPBD analogs based on structural similarity, and compared these compounds for MMP-9 repression. We identified 8 compounds similar to MPBD (Fig. 7A), none of which was contained in the DIVERSet chemical library previously screened (Fig. 2). We thus treated the HT/MMP9/F55 cells with these small molecules and measured the luciferase activity. Two compounds (7933942 and 7951841) repressed the MMP-9 promoter (Fig. 7B) with little toxicity as evident with MTT assays (Fig. 7C). Although the extent of reduction was smaller than that achieved with MPBD, 20 μM of these two compounds inhibited RANKL-induced MMP-9 expression in RAW264.7 cells (Fig. 7D) while compound 793942 inhibited PMA-induced MMP-9 expression in UMSCC-1 cells as well (Fig. 7E). These studies point to a number of structural features necessary for repression. First and foremost, the methyl group at the benzimidazole ring is likely important for inhibition of MMP-9 expression, since compound 5129073 (PBD) that lacks this group failed to repress the transgenic promoter and MMP-9 expression (Fig. 7B, D and E). However, the proximity of this group to the ring structure appears requisite since its displacement with an ethoxy group (compound 511260) generates a null compound. Nevertheless, the ring position of the methyl group however did not appear critical as evidenced by the efficacy of 7933942 in repressing MMP-9 expression. Likewise, transfer of the methyl group to the phenyl ring combined with the addition of a

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second methyl group (compound 7951841) yielded a molecule partially effective in suppressing MMP-9 expression at least in RANKL-stimulated RAW 264.7 cells. We thus conclude that MPBD serves as a lead compound for the development of therapeutic reagents treating MMP-9-dependent diseases (e.g. cancer, arthritis).

Discussion

High-throughput screening of chemical libraries using bioluminescence gene reporting technology is an innovative approach to search for small molecules capable of rectifying aberrant gene expression in cancer. However, frequent silencing of reporter genes by epigenetic mechanisms (Wakimoto, 1998; Yan and Boyd, 2006) hinders wide applications of this drug discovery strategy. In this study, a Flp-mediated homologous recombination system (Yan et al., 2004) has been exploited to readily generate cells that carry the reporter in an open chromosomal location thereby overcoming silencing effects imposed by surrounding chromatin. We have successfully demonstrated the utility of such a HTS system to identify a small molecule antagonist of MMP-9 expression. Thus, the Flp-mediated homologous recombination system serves as a useful technology to develop HTS for lead compounds that restore normal gene expression in cancer cells. Indeed, in addition to the inhibitors of MMP-9 expression, we have also identified compounds capable of repressing expression of an apoptosis suppressor c-FLIP and a p53 antagonist MDM4 (unpublished data), further demonstrating the versatility of this novel system.

MMP-9 has multiple roles in cancer biology participating in both early and late events in the cancer progression spectrum (Van den Steen et al., 2002; Hjertner et al., 2005) and therefore drugs that interfere with its expression could be of therapeutic benefit. For example, recent studies have

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implicated this metalloproteinase in the early stages of lung colonization (Acuff et al., 2006) and it can be envisaged that drugs interfering with MMP-9 expression could benefit patients at high risk for tumor dissemination. In this regard, these drugs could be alternatives to MMP-9 enzymatic inhibitors that exhibited severe side effects in clinical trials while these side effects were presumably caused by their wide spectrum of activity across the metalloproteinase family (Coussens et al., 2002). Given that regulation of transcription is a result of interplay of multiple transcription factors (Yan and Boyd, 2007), drugs repressing MMP-9 expression might be more specific and thus their side effects could be minimized. Secondly, there is a substantial body of evidence suggesting that tumor-driven bone destruction with osteolytic cancers is MMP-9-dependent (Brown et al., 2005; Dong et al., 2005). Indeed, our data clearly demonstrate the ability of MPBD to counter RANKL-induced MMP-9 expression, pre-osteoclast migration and osteoclast activity raising the possibility that anti-MMP-9 agents could be of therapeutic utility in managing this phase of the disease. Such agents could be used to complement the use of bisphosphonates which are only partially effective in countering bone resorption with osteolytic cancers such as breast and multiple myeloma (Brown et al., 2005) and sometimes have debilitating side effects including osteonecrosis of the jaw (Migliorati et al., 2005; Dunstan et al., 2007).

How does MPBD, a benzimidazole derivative, repress MMP-9 expression? Most likely, given its potent activity in our reporter assays, this compound targets MMP-9 transcription. Indeed, due to structural similarity to purine bases, it might be that the benzimidazole ring binds the minor groove of DNA to regulate transcription (Briehn et al., 2003; Chenoweth et al., 2007). However, we found no evidence that MPBD affects DNA-protein complex formation at the MMP-9 AP-1 motif, while such a mechanism would not explain how the *trans*-activation activity of the Gal4-c-Jun fusion protein is

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repressed, either. Given that MPBD inhibits the ability of the DNA-bound c-Jun to *trans*-activate transcription, it might be that this compound interferes with the interaction of c-Jun with the basal transcription factors an event essential for promoter *trans*-activation (Papavassiliou, 1998). One possibility is that MPBD blocks cell signaling that leads to phosphorylation of c-Jun, since the latter event enhances the interplay of transcription factors (Bogoyevitch and Kobe, 2006). However, this mechanism is not supported by our observations that the phosphorylation levels of c-Jun remain unchanged upon MPBD treatment. Most likely, MPBD directly binds to the AP-1 protein and affects its interactions with one or more components of the basal transcription machinery (Papavassiliou, 1998).

If MPBD targets AP-1, it raises the question as to how MMP-1, which also harbors such a motif (Yan and Boyd, 2007), was relatively unaffected in its expression. It may be that chromatin (epigenetic) factors associated with the MMP-1 promoter blunt the response of the promoter to altered AP-1 activity. Alternatively, the MMP-1 promoter may lack (a) other regulatory element(s) present in the MMP-9 regulatory sequence that is(are) required for repression. Indeed, our observation that substitution of the AP-1 binding site only partially impaired the inhibitory effect is consistent with the notion that MPBD targets multiple *cis* elements. Therefore, our finding that MPBD inhibits MMP-9 expression by antagonizing the AP-1 function adds a new function to the benzimidazole family of compounds that also inhibit the activity of casein kinase II, topoisomerase I, or PPARγ at a concentration range similar to MPBD (Rangarajan et al., 2000; Fujimura et al., 2005; Tapia et al., 2006).

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Our finding that MPBD failed to inhibit the basal MMP-9 expression although it strongly antagonized the AP-1 function and reduced the transgenic MMP-9 promoter activity deserves some discussion. The most likely possibility is that under basal conditions transcription is low with protein levels largely reflecting the prolonged half life of the transcript (~ 19h) (Sehgal and Thompson, 1999). Thus, a drug targeting transcription of the MMP-9 gene would have little repressive effect under these conditions. Alternatively, it may be that the endogenous MMP-9 promoter in quiescent cells is assembled into relatively-condensed chromatin (Yan and Boyd, 2007) that renders it unresponsive to altered AP-1 activity. In contrast, PMA-induced MMP-9 expression is accompanied by recruitment of chromatin remodeling motors to the promoter (Ma et al., 2004) which may confer responsiveness to changes in AP-1 activity. Indeed, under these latter conditions, MPBD suppressed MMP-9 expression. This latter scenario is given further weight by the observation that the extent to which the transgenic promoter is repressed in the relatively-condensed F8 chromosomal location (unpublished data) is smaller than in the open F55 location (Yan and Boyd, 2006) (Fig 5).

In summary, we have established a bioluminescence-based high-throughput screening system that overcomes reporter-gene silencing induced by flanking chromatin. Using this system, we have identified a benzimidazole derivative repressive for MMP-9 expression and consequently inhibiting cell invasion, pre-osteoclast migration and osteoclastic activity. This novel compound could serve as a prototype for a new class of anti-MMP-9 therapeutic agents benefiting patients with early disease but at high risk for tumor dissemination or for more effectively managing cancer-driven bone destruction.

Acknowledgments

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Molecular Pharmacology Fast Forward. Published on December 7, 2007 as DOI: 10.1124/mol.107.042606
This article has not been copyedited and formatted. The final version may differ from this version.

References

Acuff H, Carter K, Fingleton B, Gorden D and Matrisian L (2006) Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment. *Cancer Res* 66**:** 259-266.

Ash CS, Nason RW, Abdoh AA and Cohen MA (2000) Prognostic implications of mandibular invasion in oral cancer. *Head Neck* 22**:** 794-798.

Barski A, Cuddapah S, Cui K, Roh T, Schones D, Wang Z, Wei G, Chepelev I and Zhao K (2007) High-resolution profiling of histone methylations in the human genome. *Cell* 129**:** 823-837.

Bogoyevitch MA, Kobe B (2006) Uses for JNK: The many and varied substrates of the c-Jun N-terminal kinases. *Microbiol Mol Biol Rev* 70**:** 1061-1095

Briehn C, Weyermann P and Dervan P (2003) Alternative heterocycles for DNA recognition: the Benzimidazole/imidazole pair. *Chem Eur J* 9**:** 2110-2122.

Brown JE, Cook RJ, Major P, Lipton A, Saad F, Smith M, Lee K, Zheng M, Hei Y and Coleman RE (2005) Bone turnover markers as predictors of skeletal complications in prostate cancer, lung cancer, and other solid tumors. *J Natl Cancer Inst* 97**:** 59-69.

Chenoweth D, Poposki J, Marques M and Bervan P (2007) Programmable oligomers targeting 5'-GGGG-3' in the minor groove of DNA and NF-κB binding inhibition. *Bioorg Med Chem* 15**:** 759-770.

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: Trial and tribulations. *Science* 295**:** 2387-2392

Dong Z, Bonfil R, Chinni S, Deng X, Fiho J, Bernardo M, Vaishampayan U, Chen M, Sloane B, Sheng S, Fridman R and Cher M (2005) Matrix metallproteinase activity and osteoclasts in experimental prostate cancer bone metastasis tissue. *Am J Pathol* 166**:** 1173-1186.

Dunstan CR, Felsenberg D and Seibel MM (2007) Therapy Insight: the risks and benefits of bisphosphonates for the treatment of tumor-induced bone disease. *Nature Clinical Practice Oncology* 4**:** 42-55.

Fujimura T, Sakuma H, Konishi S, Oe T, Hosogai N, Kimura C, Aramori I and Mutoh S (2005) FK614, a novel peroxisome proliferator-activated receptor γ modulator, induces differential transactivation through a unique ligand-specific interaction with transcriptional coactivators. *J Pharmacol Sci* 99**:** 342-352.

Guise TA, Kozlow WM, Heras-Herzig A, Padalecki SS, Yi JJ and Chirgwin JM (2005) Molecular mechanisms of breast cancer metastases to bone. *Clinical Breast Cancer Supplement* 5**:** S46-S53.

Hjertner O, Standal T, Borset M, Sunda A and Waage A (2005) Identification of new targets for therapy of osteolytic bone disease in multple myeloma. *Current Drug Targets* 6**:** 701-711.

Huang Q, Raya A, DeJesus P, Chao S, Quon KC, Caldwell J, Chanda S, Zpisua-Belmonte J and Schultz P (2004) Identification of p53 regulators by genome-wide functional analysis. *Proc Natl Acad Sci USA* 101**:** 3456-3461.

Juarez J, Clayman G, Nakajima M, Tanabe K, Saya H, Nicolson G and Boyd D (1993) Role and regulation of 92 kDa type IV collagenase (MMP-9) in invasive squamous cell carcinoma of the oral cavity. *Int J Cancer* 54**:** 73-80.

Ma Z, Shah R, Chang M and Benveniste E (2004) Coordination of cell signaling, chromatin remodeling, histone modifications, and regulator recruitment in human matrix metalloproteinase 9 gene transcription. *Mol Cell Biol* 24**:** 5496-5509.

Migliorati CA, Schubert MM, Peterson DE and Seneda LM (2005) Bisphosphonate-associated osteonecrosis of mandibular and maxillary bone. *Cancer* 104**:** 83-93.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity Assays. *J Immunol Meth* 65**:** 55-63.

Pandolfi P (2001) Transcription therapy for cancer. *Oncogene* 20**:** 3116-3127.

Papavassiliou AG (1998) Transcription-factor-modulating agents: Precision and selectivity in drug design. *Mol Med Today* 4**:** 238-366.

Rangarajan M, Kim J, Sim S-P, Liu A, Liu L and LaVoie E (2000) Topoisomerase I inhibition and cytotoxicity of 5-bromo- and 5-phenylterbenzimidazoles. *Bioorg Med Chem* 8**:** 2591-2600.

Rapisarda A, Uranchimeg B, Scudiero D, Selby M, Sausville E, Shoemaker RH and Melillo G (2002) Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. *Cancer Res* 62**:** 4316-4324.

Sehgal I and Thompson T (1999) Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor-β1 in human prostate cancer cell lines. *Mol Biol Cell* 10**:** 407-416.

Shin M, Yan C and Boyd D (2002) An inhibitor of c-jun aminoterminal kinase (SP600125) represses c-Jun activation, DNA-binding and PMA-inducible 92-kDa type IV collagenase expression. *Biochim Biophys Acta* 1589**:** 311-316.

Shoemaker R, Scudiero D, Melillo G, Currens M, Monks A, Rabow A, Covell D and Sausville E (2002) Application of high-throughput, molecular-targeted screening to anticancer drug discovery. *Curr Topics Med Chem* 2**:** 229-246.

Sobell H (1985) Actinomycin and DNA transcription. *Proc Natl Acad Sci USA* 82**:** 5328-31.

Sternlicht M and Werb Z (2001) How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 17**:** 516.

Sundaram K, Nishimura R, Senn J, Youssef R, London S and Reddy S (2007) RANK ligand signaling modulates the matrix metalloproteinase-9 gene expression during osteoclast differentiation. *Exp Cell Res* 313**:** 168-178.

Tapia J, Torres V, Rodriguez D, Leyton L and Quest A (2006) Casein kinase 2 (CK2) increases survivin expression via enhanced ß-catenin-T cell factor/lymphoid enhancer binding factor-dependent transcription. *Proc Natl Acad Sci USA* 103**:** 15079-15084.

Van den Steen P, Dubois B, Nelissen I, Rudd P, Dwek R and Opdenakker G (2002) Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol* 37**:** 375-536.

Molecular Pharmacology Fast Forward. Published on December 7, 2007 as DOI: 10.1124/mol.107.042606
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Van Valckenborgh E, Croucher PI, De Raeve H, Carron C, De Leenheer E, Blacher S, Devy L, Noel A, De Bruyne E, Asosingh K, Van Riet I, Van Camp B and Vanderkerken K (2004) Multifunctional role of matrix metalloproteinases in multiple myeloma. *American Journal of Pathology* 165**:** 896-878.

Wakimoto B (1998) Beyond the nucleosome: epigenetic aspects of position-effect variegation in Drosophila. *Cell* 93**:** 321-324.

Wang W, Kim S and El Deiry W (2006) Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. *Proc Natl Acad Sci USA* 103**:** 11003-11008.

Yan C and Boyd D (2006) Histone H3 acetylation and H3 K4 methylation define distinct chromatin regions permissive for transgene expression. *Mol Cell Biol* 26**:** 6357-6371.

Yan C and Boyd D (2007) Regulation of matrix metalloproteinase gene expression. *J Cell Physiol* 211**:** 19-26.

Yan C, Wang H, Aggarwal B and Boyd D (2004) A novel homologous recombination system to study 92 kDa type IV collagenase transcription demonstrates that the NF-κB motif drives the transition from a repressed to an activated state of gene expression. *FASEB J* 18**:** 540-541.

Yan C, Wang H and Boyd D (2001) *KiSS-1* represses 92-kDa type IV collagenase expression by down-regulating NF-κB binding to the promoter as a consequence of IκBα-induced block of p65/p50 nuclear translocation. *J Biol Chem* 276**:** 1164-1172.

Zhang J-H, Chung TY, Oldenburg KR (1999) A simple statistical parameterfor use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 4**:** 67-73.

Molecular Pharmacology Fast Forward. Published on December 7, 2007 as DOI: 10.1124/mol.107.042606
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

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Legends for Figures:

Fig. 1. Development of a novel system for high-throughput screening for small MMP-9 transcription regulators. A, Schematic showing recombination between FRT fragments allowing for integration of a luciferase reporter gene driven by a MMP-9 promoter into an open chromosomal location (the F55 site). **B,** HT/MMP9/F55 cells were plated into a 96-well plate at indicated densities, treated with PMA for 5 h, and lysed for luciferase activity assays. **C,** HT/MMP9/F55 cells (1.5×10^4/well) were plated into a 96-well plate, treated with indicated amounts of Actinomycin D overnight, and lysed for luciferase activity assays. **D,** The transgenic cells cultured in 96-well plates were treated with 1 μM of Actinomycin D (positive control, 2 plates) or DMSO (negative control, 1 plate) overnight, and lysed for luciferase activity assays. The assay results from the two positive-control plates were combined and used to calculate Z′ as described previously (Zhang et al., 1999). **E,** The transgenic cells were treated with 5 μM of 240 randomly-selected compounds overnight and lysed for luciferase activity assays. Results from two independent experiments were used to generate a Q-Q plot to determine the reproducibility of the screening assays.

Fig. 2. Identification of small molecules repressive for transgenic MMP-9 promoter. A, HT/MMP9/F55 cells were plated into 96-well plates and treated overnight with 5μM of compounds from a diversity-set chemical library. Cell lysates were prepared for luciferase activity assays. The relative luciferase activities were converted into logarithm values (binary logarithm, i.e., $\log_2$) and plotted for each compound. For compounds that inhibited luciferase activity more than 75%, average values from two independent assays were used for analyses. **B and C,** The cells were treated with the indicated amounts of the 4 compounds for 5 h, and then lysed for luciferase activity (B) or MTT assays as described (Mosmann, 1983) (C).

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Fig. 3. A benzimidazole derivative inhibits MMP-9 expression in cancer cells. A, HT1080 cells were treated with the indicated amounts of MPBD or DMSO for 24 h followed by addition of 50 nM PMA or DMSO into the medium. Condition media were collected and subjected to zymography. **B, C, and D,** HT1080 cells were treated as in (A). Total RNA was prepared, reverse transcribed, and cDNA subjected to real-time PCR assays for MMP-9 (B), MMP-2 (C), MMP-1 (D) and β-actin mRNA amounts. The β-actin mRNA amounts were used to normalize loaded cDNA amounts. Data are depicted as average ± SD values of 3 determinations. **, $p < 0.01$ versus PMA alone (Student *t*-test).

Fig. 4. MPBD inhibits invasion of UMSCC-1 oral cancer cells. A, UMSCC-1 cells were treated with indicated concentrations of MPBD or DMSO for 24 h, and then cultured in serum-free medium (containing the compound) with 50 nM PMA for 24 h. Conditioned media from equal number of cells were used for zymography. **B,** Cells were treated with 20 μM MPBD or DMSO for 24 h followed by addition of 50 nM PMA into the medium. 24 h later, cells were plated into Transwells coated with Matrigel and allowed to invade through the reconstituted basement membrane for 20 h after which invaded cells were stained and quantified. Numbers indicates relative invasive capability. ***, $p < 0.001$ versus the DMSO control (Student *t*-test).

Fig. 5. MPBD inhibits MMP-9 expression, pre-osteoclast migration and osteoclastic activity. A, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 24 h followed by addition of the indicated amounts of MPBD or DMSO. 24 h later, cells were placed in serum-free medium and cultured for another 24 h. Conditioned media were collected and subjected to zymography after normalization for any cell number differences. **B,** RAW264.7 cells were stimulated with 100 ng/ml

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RANKL for 4 days and MPBD was added into the medium in the 3rd day of the treatments. The stimulated cells and control cells were then stained for TRAP activity. **C,** RAW264.7 cells were stimulated with 100 ng/ml RANKL for 24 h followed by addition of 20 μM MPBD, PBD or DMSO into the culture medium. 48 h later, cells were plated into Transwells, and the latter were inserted into a 24-well plate containing UMSCC-1 oral cancer cells. Migratory cells were stained and counted after 40 h of incubation. ***, p <0.001, Student *t*-test. **D,** RAW264.7 cells were stimulated with 100 ng/ml RANKL for 3 days and then transferred to 16-well BioCoat Osteologic slides containing 20 μM PBD or DMSO. Cells were removed by Bleach 3 days later and the slides photographed (Magnification-40X). **, p <0.01 versus RANKL alone (Student *t*-test). **E,** RAW264.7 cells were treated as (d) and osteoclasts were stained for TRAP.

Fig. 6. MPBD antagonizes AP-1 function. A, HT1080 cells harboring wild-type, the proximal AP-1-site-mutated, or NFκB-site-mutated MMP-9 promoter were treated with the indicated amounts of MPBD for 5 h, and then lysed for luciferase activity assays. *, p <0.05, **, p <0.01 versus wild-type promoter (Student *t*-test). **B, D,** HT1080 cells were plated in 96-well plates, transfected with pAP-1-luc for 24 h, and then treated with indicated amounts of MPBD or DMSO overnight. The cells were lysed for luciferase activity assays (B) or Western blotting (D). *, p <0.05, **, p <0.01 versus DMSO control (Student t-test). **C, E,** A luciferase reporter containing Gal4-binding site (pFR-luc) was co-transfected with a construct (pFA c-Jun) expression a Gal4-c-Jun fusion protein, or the empty vector (pFc-dbd), into HT1080 cells. 24 h later, the cells were treated with indicated amounts of MPBD for 16 h and then lysed for luciferase activity assays (C) or Western blotting (E). For the JNK-inhibition control, the cells were treated with 20 μM of SP600125 for 16 h. **, p <0.01 versus DMSO control (Student *t*-test). Data are depicted as average ± SD values of 3 independent experiments.

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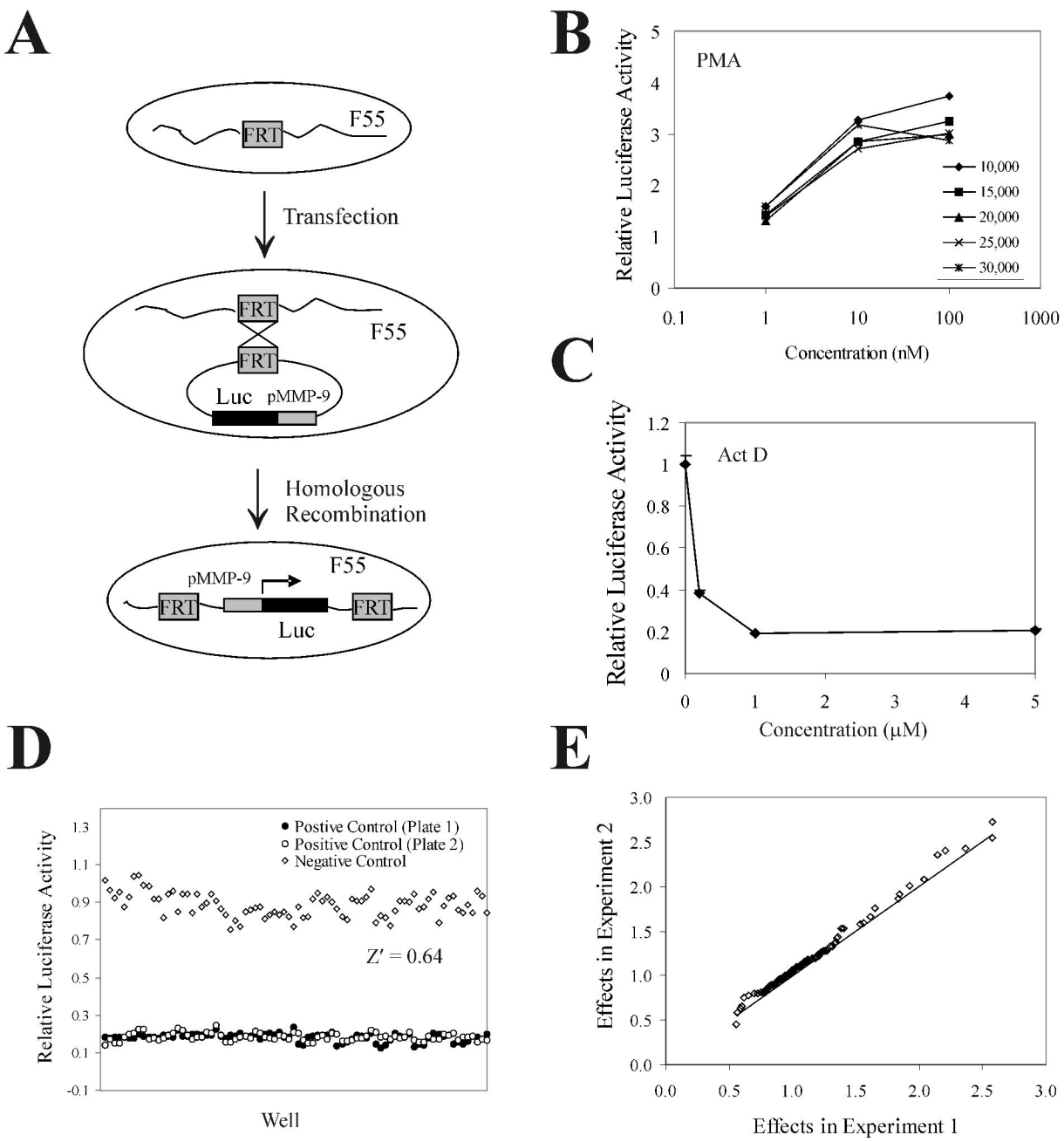
Fig. 7. Structure-Activity Studies with MPBD analogs. A, Structures of MPBD and its analogs. **B and C,** HT/MMP9/F55 cells were treated with the indicated amounts of analogs for 5 h and then lysed for luciferase activity assays (B) or MTT assays (C). *, p <0.05, **, p <0.01 versus DMSO control (Student *t*-test). **D,** UMSCC-1 cells were treated with 20 μM MPBD or its analogs as in Fig 4A, and conditioned media subjected to zymography after normalization for cell number differences. **E,** RAW264.7 cells were treated with 20 μM MPBD or its analogs as in Fig 5A for gelatin zymography. For Panels B and C, data are shown as average $\pm$ SD values of 3 separate determinations.

Figure 1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F55
FRT
Transfection
FRT
F55
FRT
Luc pMMP-9
Homologous
Recombination
pMMP-9
F55
FRT
FRT
Luc
B
PMA
Relative Luciferase Activity
10,000
15,000
20,000
25,000
30,000
Concentration (nM)
C
Act D
Relative Luciferase Activity
Concentration (μM)
D
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Positive Control (Plate 2)
Negative Control
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Relative Luciferase Activity
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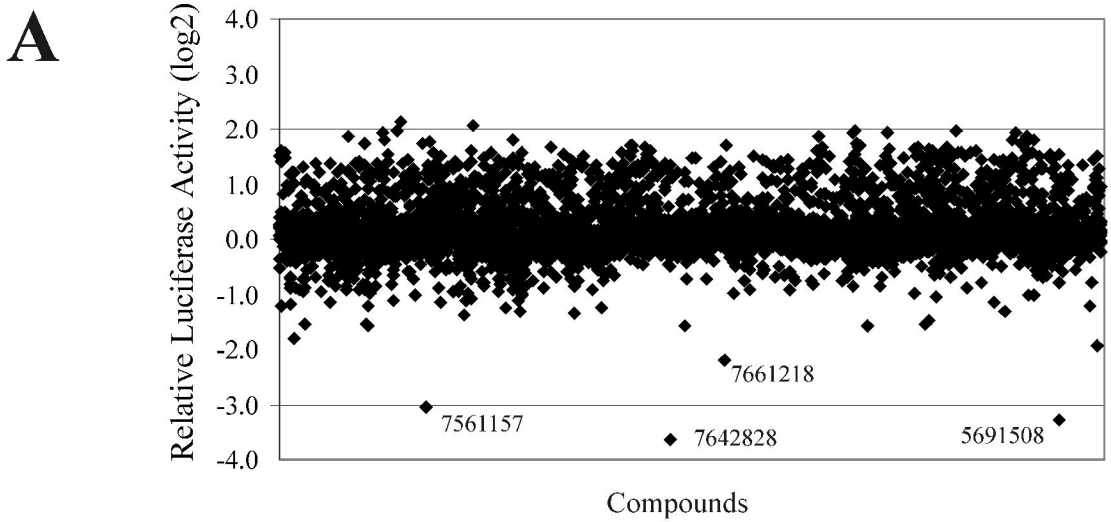
E
Effects in Experiment 2
Effects in Experiment 1

Figure 2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2.0
1.0
0.0
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-2.0
-3.0
-4.0
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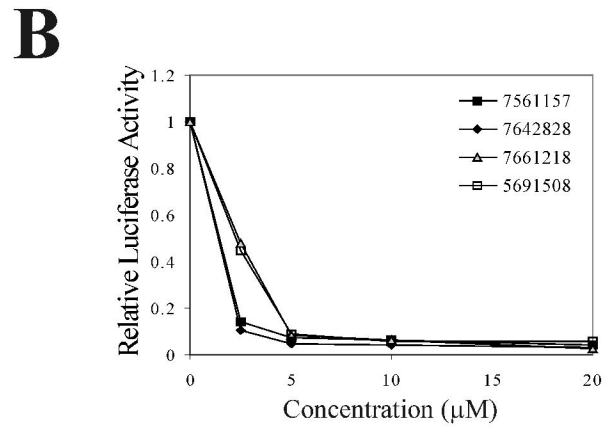
7561157
7642828
5691508
Compounds


B
Relative Luciferase Activity
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1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
Concentration (μM)
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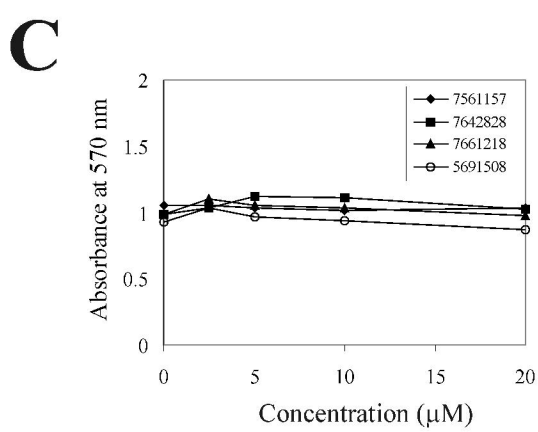
7642828
7661218
5691508


C
Absorbance at 570 nm
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1
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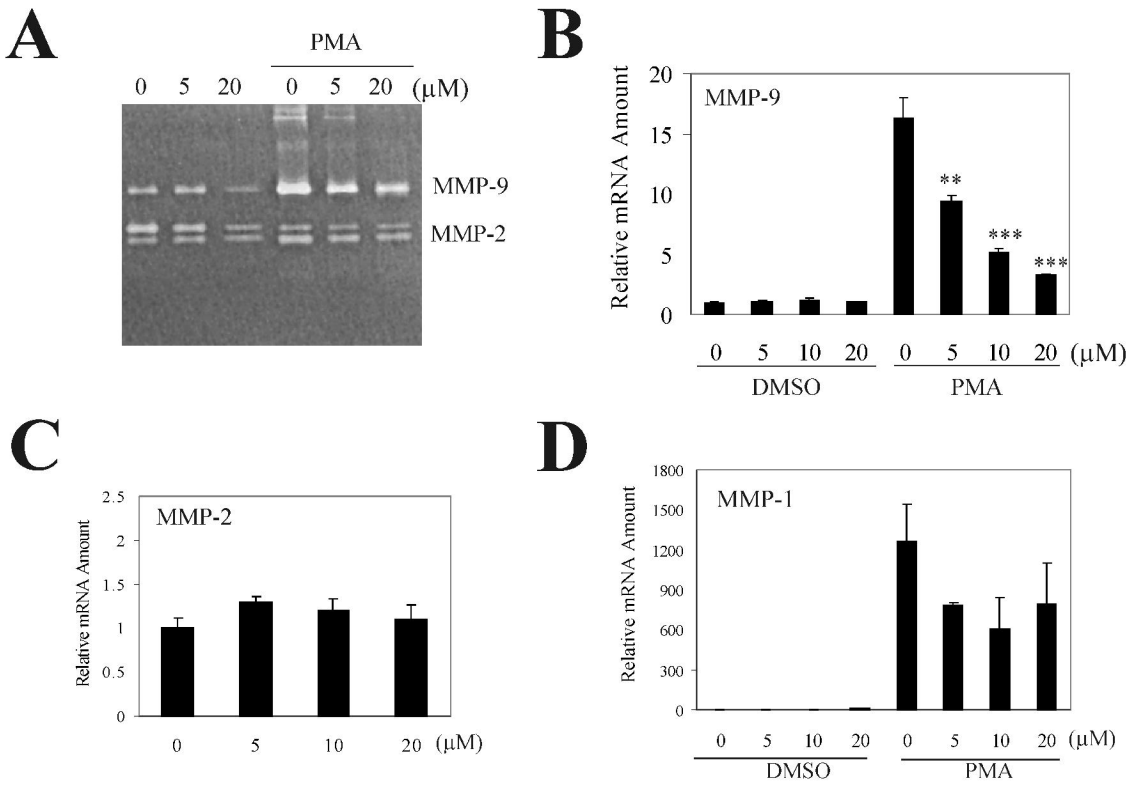
5
10
15
20
Concentration (μM)
7561157
7642828
7661218
5691508

Figure 3


A
PMA
0 5 20 0 5 20 (μM)
MMP-9
MMP-2
B
MMP-9
Relative mRNA Amount
20
15
10
5
0
**
***
***
0 5 10 20 0 5 10 20 (μM)
DMSO
PMA
C
MMP-2
Relative mRNA Amount
2.5
2
1.5
1
0.5
0
0 5 10 20 (μM)
D
MMP-1
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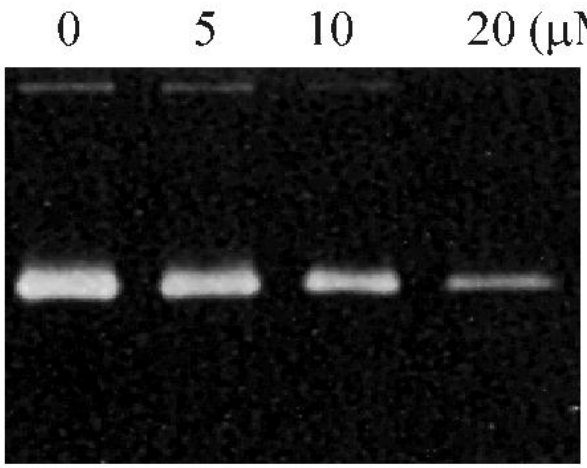
1500
1200
900
600
300
0
0 5 10 20 0 5 10 20 (μM)
DMSO
PMA

Figure 4

A



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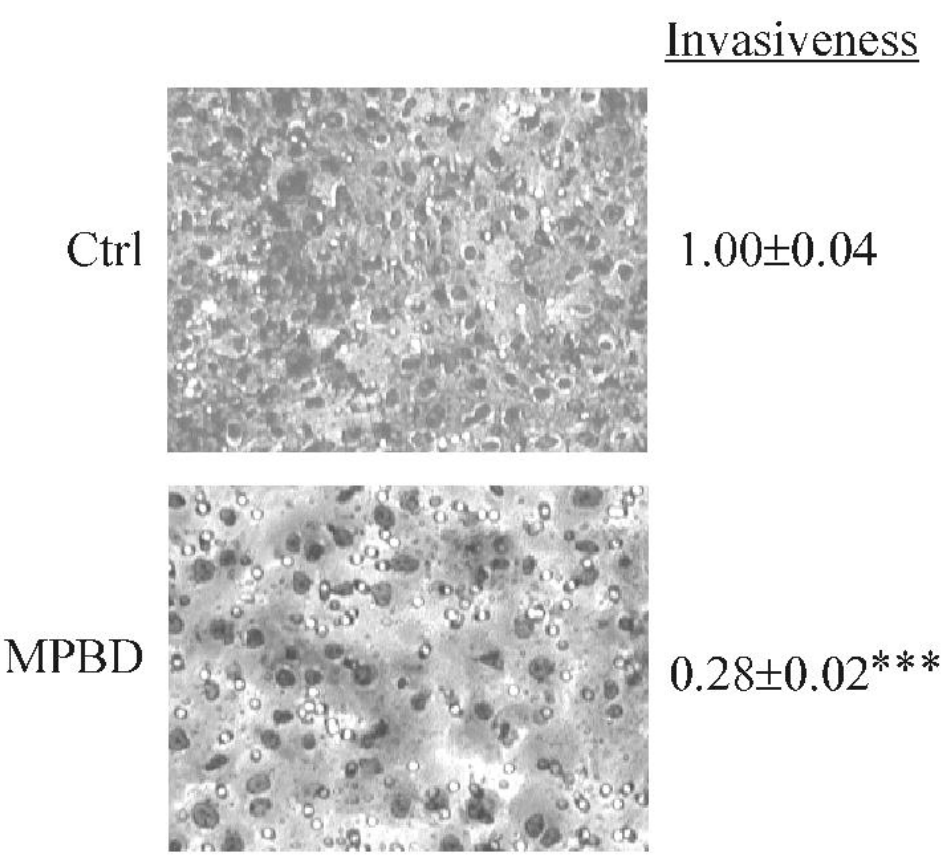


Figure 5

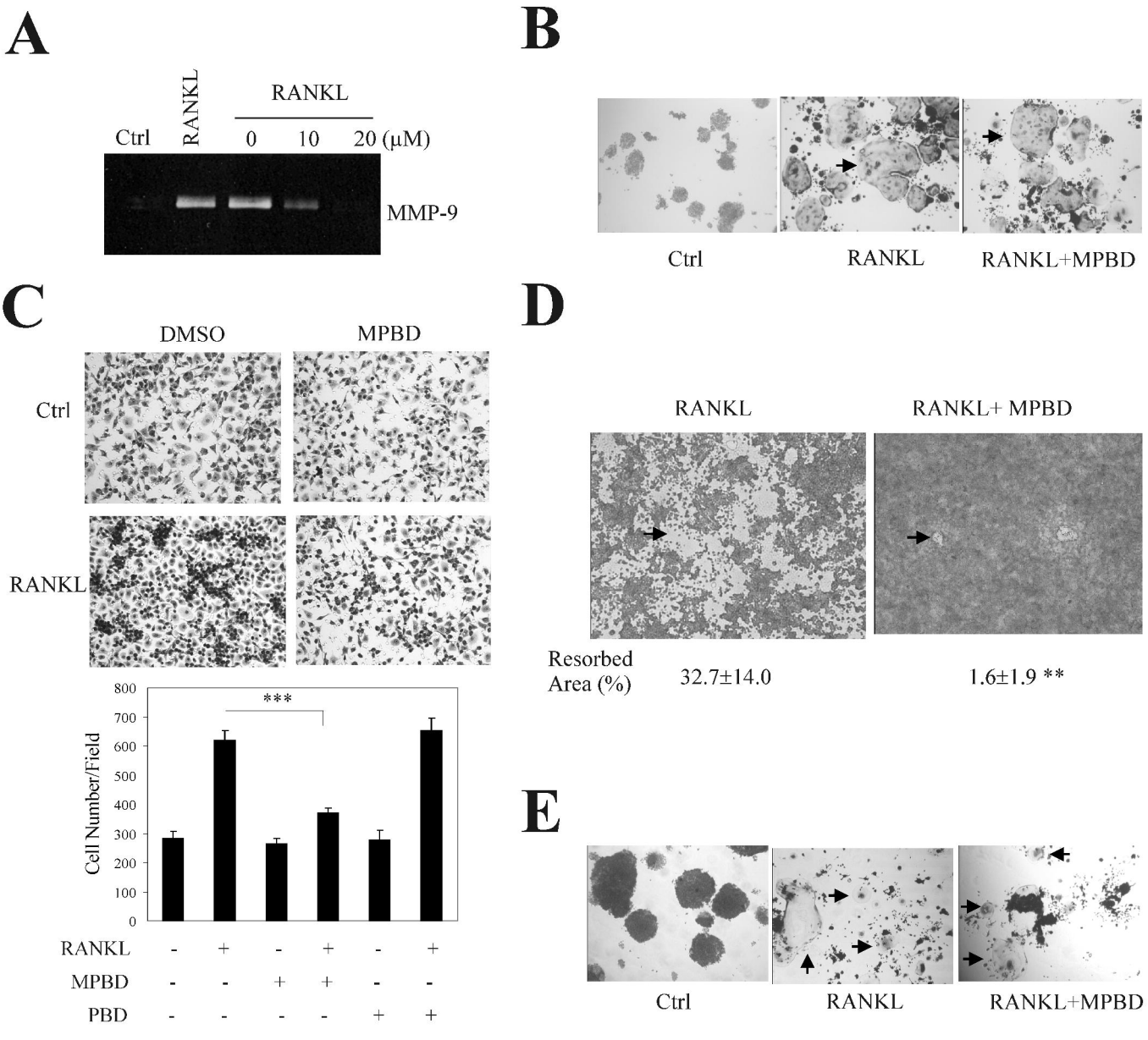
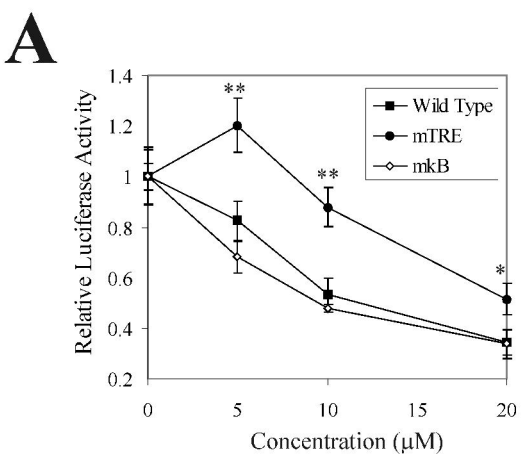
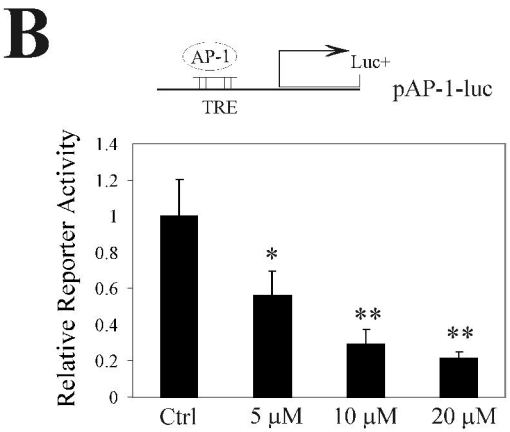
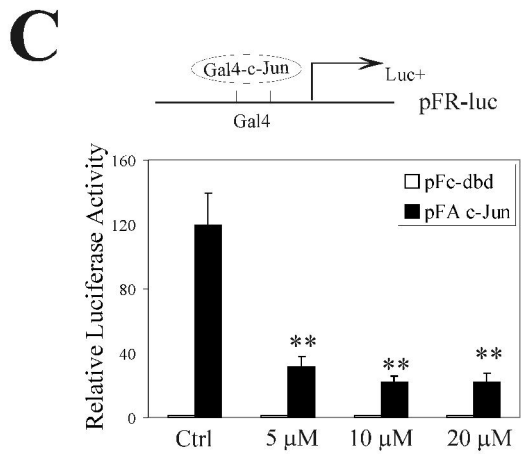
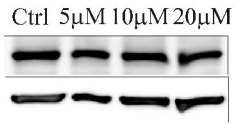

A
RANKL
RANKL
Ctrl
0
10
20 (μM)
MMP-9
B
Ctrl
RANKL
RANKL+MPBD
C
DMSO
MPBD
Ctrl
RANKL
800
700
600
500
400
300
200
100
0
Cell Number/Field
***
RANKL - + - + - +
MPBD - - + + - -
PBD - - - - + +
D
RANKL
RANKL+ MPBD
Resorbed Area (%)
32.7±14.0
1.6±1.9 **
E
Ctrl
RANKL
RANKL+MPBD

Figure 6


A
Relative Luciferase Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5
10
15
20
Concentration (μM)
Wild Type
mTRE
mkB
**
**
*


B
AP-1
Luc+
TRE
pAP-1-luc
Relative Reporter Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
Ctrl
5 μM
10 μM
20 μM


C
Gal4-c-Jun
Luc+
Gal4
pFR-luc
Relative Luciferase Activity
160
120
80
40
0
pFc-dbd
pFA c-Jun
**
**
**
Ctrl
5 μM
10 μM
20 μM


D
Ctrl 5μM 10μM 20μM
p-c-Jun
β-actin

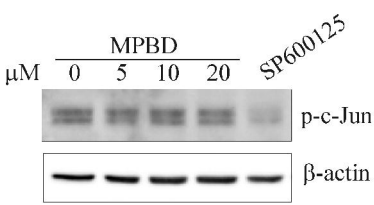
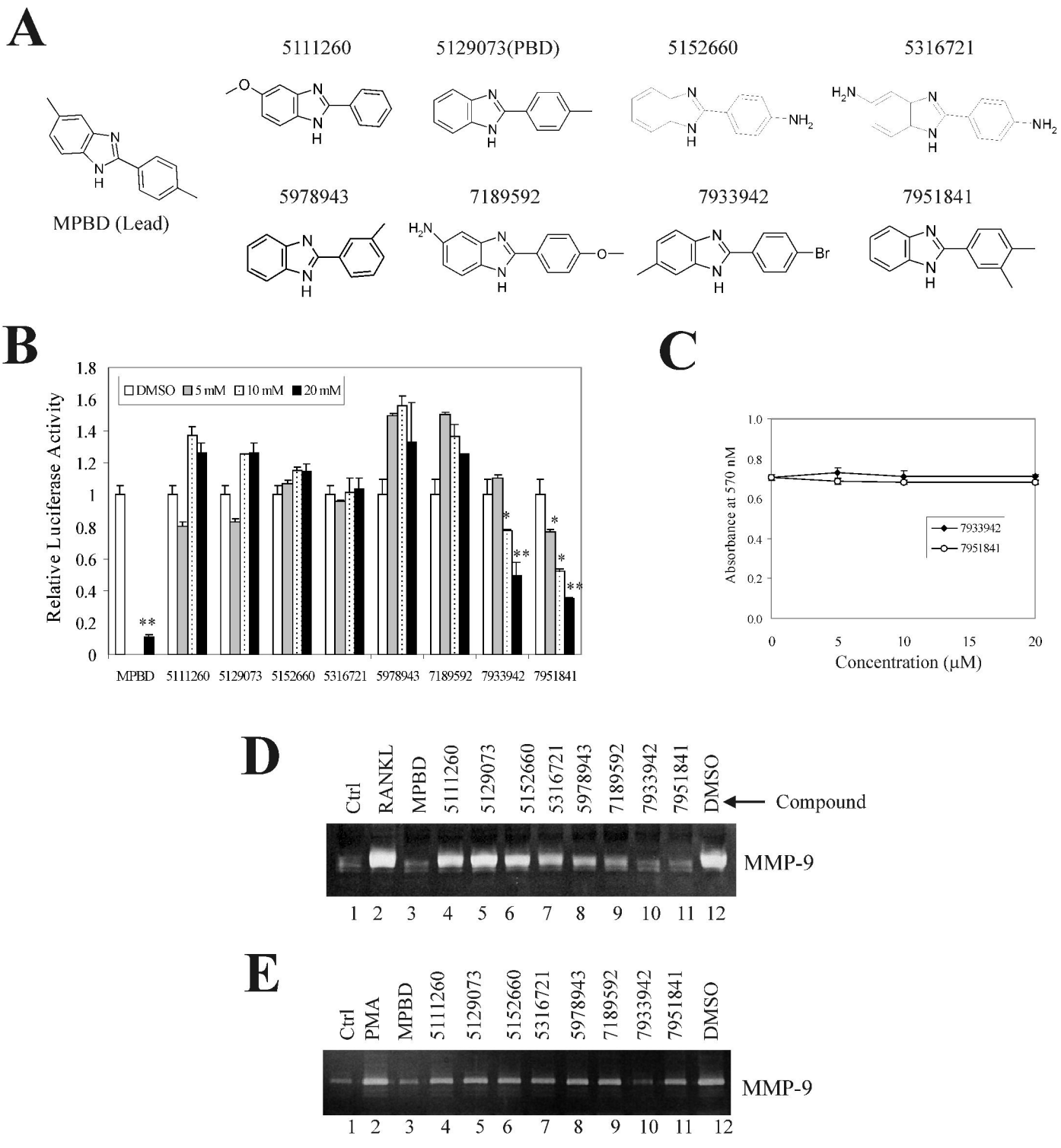

E
MPBD
μM 0 5 10 20
SP600125
p-c-Jun
β-actin

Figure 7


A
MPBD (Lead)
5111260
5129073(PBD)
5152660
5316721
5978943
7189592
7933942
7951841
B
Relative Luciferase Activity
DMSO
5 mM
10 mM
20 mM
MPBD
5111260
5129073
5152660
5316721
5978943
7189592
7933942
7951841
C
Absorbance at 570 nM
Concentration (μM)
7933942
7951841
D
Ctrl
RANKL
MPBD
5111260
5129073
5152660
5316721
5978943
7189592
7933942
7951841
DMSO
Compound
MMP-9
1 2 3 4 5 6 7 8 9 10 11 12
E
Ctrl
PMA
MPBD
5111260
5129073
5152660
5316721
5978943
7189592
7933942
7951841
DMSO
MMP-9
1 2 3 4 5 6 7 8 9 10 11 12