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A p38 mitogen-activated protein kinase inhibitor screening method using growth recovery of *Escherichia coli* as an index

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ABSTRACT

The p38 mitogen-activated protein (MAP) kinase is the central signaling molecule regulating the cellular response to a multitude of external stimuli. Thus, inhibitors of this enzyme are postulated to have significant therapeutic potential for the treatment of some diseases, especially where aberrant cytokine signaling is the driver of disease. Here we established a simple inhibitor screening method for a human protein by using bacteria in combination with the growth recovery as an index. The screening successfully identified benzyl coumarin derivatives as p38 inhibitors. These compounds not only rescue growth retardation of p38-transformed bacteria but also inhibit p38 activity in vitro and in human cells. This study demonstrates that this is a promising and economical inhibitor screening method not only for p38 but also for other proteins.

The p38 mitogen-activated protein (MAP)[1] kinase is identified as either an antiinflammatory drug binding protein, a lipopolysaccharide (LPS)-activated protein kinase, or a stress-responsive protein kinase [1–3]. It is implicated as a mediator to transmit intracellular signaling for cell survival, differentiation, and response to stress [4,5]. p38 MAP kinase also plays important roles in immune responses [1] and is a target of drug for inflammatory diseases [6,7].

The key to facilitating drug development and discoveries is using efficient, ideally economical, and generally applicable screening methods. Although there are many sophisticated screening methods to date, and they have contributed tremendously to society, many patients still suffer from diseases caused by known or unknown mechanisms. To accelerate the research and development of drugs and/or treatments, we need efficient approaches with new ideas.

Here we report the establishment of a simple screening method based on a conventional screening using bacteria with a new concept. Using the described method, chemical libraries were screened for the p38 inhibitor and benzyl coumarin derivatives were identified as promising candidates. These benzyl coumarin derivatives inhibited p38 activities in vitro and in human cervical cancer (HeLa) cells. Our results demonstrate that benzyl coumarin can be an alternate core structure for the development of additional p38 inhibitors [8]. Moreover, the rationale of this screening method with transformed bacteria can be applicable for other gene products.

Materials and methods

Plasmid construction

For the expression of human p38 protein in *Escherichia coli*, *Cla*I–*Apa*I fragment of pcDNA3Flag–hp38 [9] was cloned into pRSET–CA plasmid in which new *Cla*I and *Apa*I sites were introduced in multicloning sites of pRSET–C (Invitrogen). Then we named the His-tagged human p38 expression plasmid as pRSET–hp38. Next, p38 mutants with a single amino acid substitution, K53A [10] and T106 M [11], were constructed by following the method described in a previous report [12]. We designed the primers in this thermal

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[1] *Abbreviations used:* MAP, mitogen-activated protein; LPS, lipopolysaccharide; LB, Luria–Bertani broth; NPDepo, Natural Products Depository; IL-1β, interleukin-1β; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MEK, MAP kinase kinase; GST, glutathione *S*-transferase; MK2, MAP kinase-activated kinase 2; 3Bn7H4 M, 3-benzyl-7-hydroxy-4-methyl coumarin; 7H4 M, 7-hydroxy-4-methyl coumarin; TNF-α, tumor necrosis factor-α.

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cycling mutagenesis: GGAGAGCTTCGCCACTGCCACACGTAACCC for K53A and TGCCCCCATGAGATGCATCACCAGATACAC for T106 M. pRSET–CA was used as a control plasmid.

Bacteria strains

E. coli BL21(DE3) were transformed with pRSET–CA or pRSET–hp38. We named these strains as BL21–CA and BL21–hp38, respectively. These strains were grown in liquid Luria–Bertani broth (LB) [13] supplemented with 100 μg/ml ampicillin prior to screening or in the screening medium containing 0.5% (w/v) bacto tryptone, 0.25% (w/v) bacto yeast extract, 0.25% (w/v) NaCl, and 100 μg/ml ampicillin.

Reagents and antibodies

Screening samples were obtained from the Natural Products Depository (NPDepo, RIKEN, Japan). Recombinant human interleukin-1β (IL-1 β) was purchased from Pepro Tech EC. Anti-His antibody was obtained from Santa Cruz Biotechnology. Anti-phospho-p38 MAPK (T180/Y182) (3D7), anti-phospho-MAPKAPK-2 (Thr334), anti-MAPKAPK-2, anti-phospho-p44/42 MAPK (Thr202/Tyr204), and anti-p44/42 MAPK antibodies were obtained from Cell Signaling. Anti-p38 antibody was prepared as described previously [14].

Primary screening

Overnight cultures of BL21–hp38 were diluted 1:1000 in screening medium, and 100 μl of aliquot was transferred to each well in a 96-well plate. Then 1 μl of the test sample was added in each well to a final concentration of 33 μg/ml, and the growth of the bacteria at 37 °C with constant shaking was monitored by measuring absorbance at 600 nm every 30 min using a newly developed bio microplate reader, HiTS, developed by Scinics (Japan). The results were obtained as growth curves as well as raw data. Dimethyl sulfoxide (DMSO) and 1 μM SB202190 were used as negative and positive controls, respectively.

Kinase assay

Bacterially produced His–hp38 and GST–ATF2(1–109) [15] were purified according to the manufacturer's instructions and used for

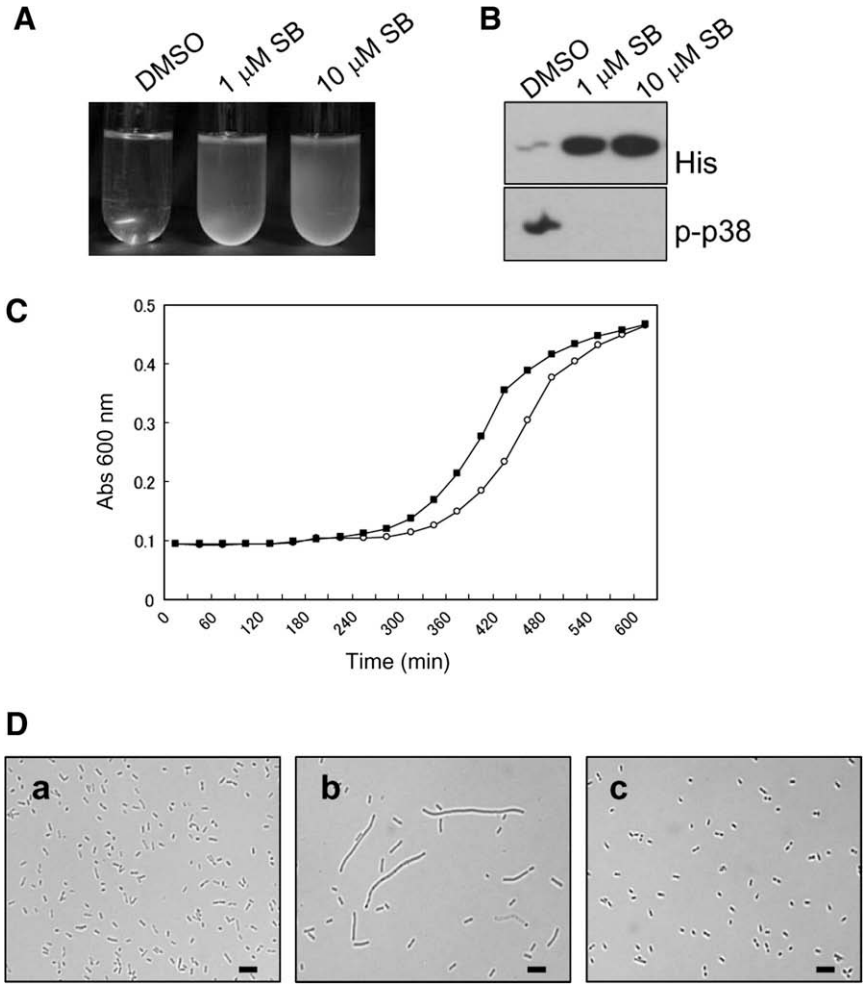

Fig. 1. Bacterial growth recovery by the administration of inhibitor. (A) Effects of the p38 inhibitor, SB202190, on *E. coli* BL21(DE3) transformed by His–hp38 expression plasmid (BL21–hp38). Increasing doses of SB202190 rescue the growth retardation of the bacteria. Overnight cultures (1:1000 dilution) were incubated for another 6 h with increasing doses of the inhibitor. (B) Western blotting using anti-His and anti-phospho p38 (p-p38) reveal that BL21-hp38 has leaky expression of p38 and p38 is phosphorylated without induction by isopropyl-thio-β-D-galactopyranoside (IPTG), which is generally used as an inducer to produce the exogenous protein, and that the phosphorylation was abolished by SB202190 treatment. (C) Growth kinetics was estimated by measuring absorbance at 600 nm every 30 min for 10 h at 37 °C. SB202190 induces the growth of the bacteria. Open circles: DMSO; filled squares: 1.0 μM SB202190. (D) Microscopic examinations reveal a typical filamentation temperature-sensitive mutant phenotype in the pRSET–hp38-transformed strain BL21–hp38 (b) and bacteria harboring the control plasmid pRSET–CA named BL21–CA (a). BL21–hp38 with SB202190 shows the wild-type phenotype similar to BL21–CA (c). Scale bar: 10 μm.