Identifying Protein Kinase Inhibitors Using an Assay Based on

Inhibition of Aerial Hyphae Formation in *Streptomyces*

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We have identified a strain of *Streptomyces* in which aerial hyphae formation appears to be especially sensitive to inhibition by protein kinase inhibitors. Using this assay, a number of bacterial cultures have been screened and novel inhibitors of eukaryotic protein kinases have been identified. Since *M. tuberculosis* possesses multiple eukaryotic-like protein kinase genes, we tested the active kinase inhibitors for the inhibition of mycobacterial growth and obtained several potent compounds. This identifies a new biochemical class of antimycobacterial agents.

In both eukaryotic and prokaryotic cells a large repertoire of regulatory systems is modulated by a variety of extracellular signals. In eukaryotic cells, the control of proliferation and differentiation is achieved by multiple signal transduction pathways, which are regulated by the co-ordinated action of protein kinases and phosphatases. The protein kinases fall primarily into two classes, those which phosphorylate tyrosine residues and those which target serine and threonine residues. Specificity of response is ensured through a variety of strategies. Unique receptors and docking proteins, compartmentalization of pathways, and specific calcium ion channels all play a role in the transmission of extracellular signals *via* kinases and phosphatases to transcription factors[1,2].

Similarly, prokaryotic cells largely rely on protein phosphorylation cascades for regulation of cellular activities, but these are primarily histidine kinases, which are part of the sensing domain of two-component regulatory systems. These kinases and their associated response regulators are involved in adaptive responses such as nitrogen fixation, chemotaxis and the regulation of sporulation in *Bacillus* species[3,4].

Recently, a number of reports indicate that eukaryotic-like kinase and phosphatase activities may complement two-component systems in several different types of bacteria. Phosphorylation of tyrosine has now been reported in a number of species, but it is usually limited to phosphorylation on a small number of proteins, such as two flagellar proteins in *Pseudomonas aeruginosa*[5] or one protein in *Mycobacterium tuberculosis*[6]. Nucleotide sequencing of the genomes of several bacteria has now revealed an abundance of such post-translational modifying enzymes. Using phosphotyrosine specific antibodies, we have shown that in several *Streptomyces* species a variety of proteins are phosphorylated on tyrosine residues[7]. Each of the five species studied exhibited a unique pattern of protein tyrosine phosphorylation and these patterns of protein phosphorylation varied during the growth phase and according to culture conditions. This led us to speculate that the complex growth cycle exhibited by these filamentous, sporulating bacteria and their diverse secondary metabolic pathways may be controlled in part by the action of protein tyrosine kinases and phosphatases. Protein tyrosine phosphorylation in *Streptomyces* has been

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confirmed in another laboratory and a protein phosphatase with phosphotyrosine specificity in *Streptomyces coelicolor* identified. This activity was also variable according to growth conditions[8]. The *S. coelicolor* sequencing project has confirmed the presence of at least a dozen serine/threonine protein kinases (D. HOPWOOD, personal communication).

Further evidence for eukaryotic-like signalling in prokaryotes has come from the study of other bacteria with complex cell cycles. Protein tyrosine phosphorylation has been demonstrated in *Myxococcus xanthus*, and this phosphorylation is involved in at least two stages of development, aggregation and sporulation[9]. Furthermore, serine/threonine kinase activities have been convincingly demonstrated in other bacteria in which cell-cell interactions take place, including *Myxococcus xanthus* and cyanobacteria: *Synechococcus* and *Anabaena*[10].

Inhibitors of kinases and their cognate phosphatases are of considerable pharmaceutical interest for a wide variety of disease targets[11,12] and numerous specific biochemical assays have been developed for high throughput screening. Some of the compounds known to inhibit eukaryotic protein kinases have been tested for effects on various developmental activities of *Streptomyces griseus*[13]. Staurosporine and compound K-252a are global inhibitors of eukaryotic protein kinases (probably because they bind at the ATP binding site in the catalytic region) and both these compounds inhibited phosphorylation of *S. griseus* proteins in *in vitro* labelling studies. Additionally, herbimicin and radicicol, thought to be specific inhibitors of tyrosine kinases, inhibited the phosphorylation of a streptomycete protein in an *in vitro* kinase assay; inhibition of tyrosine phosphorylation by these two compounds in other systems has since been shown to be an indirect effect[14]. Staurosporine and K-252a were found to inhibit the formation of aerial hyphae and spore formation, pigment production and production of the antibiotic streptomycin by *S. griseus*. Growth of vegetative mycelia was not affected. These compounds did not inhibit phosphotransfer when tested in *E. coli*. Such observations lend further support to the hypothesis that *Streptomyces* spp. employ eukaryotic-like signalling systems in developmental processes in addition to conventional prokaryotic two-component regulatory systems, and suggested to us that it might be possible to develop an assay employing streptomycetes to screen for a broad range of inhibitors of eukaryotic signal transduction. A number of streptomycetes were examined and the strain *Streptomyces* 85E with good growth and developmental characteristics on solid medium was found to be especially suited for such an

assay. Inhibitors of aerial hyphae formation can be readily detected by the appearance of "bald" colonies. Tests with several known eukaryotic kinase inhibitors such as the tyrphostins and genistein confirmed inhibition of aerial hyphal growth in the strain.

Using *Streptomyces* 85E as an assay strain for kinase inhibitors appears to identify a wide range of eukaryotic kinase modulators, presumably because the streptomycete enzymes are evolutionary forerunners of their highly specific eukaryotic counterparts. This simple assay should readily permit the identification of signal transduction inhibitors for a variety of applications including anti-infectives, antitumor agents and kinase dependent functions related to human disease. An advantage of the whole cell streptomycete assay is that it readily identifies cytotoxic activity of the compounds being tested. In addition, several of the inhibitors found using this assay were found to be effective inhibitors of mycobacteria, suggesting that protein kinases or cognate phosphatases may be useful targets for new classes of antibiotics effective for the treatment of mycobacterial infections and are thus likely to be good targets for drug development.

Materials and Methods

Strains

The following bacterial strains were used for inhibition testing: *Streptomyces* 85E ATCC 55824, *Streptomyces griseus* ATCC 23345, *Bacillus subtilis* JH642, *Staphylococcus aureus* RN450, *E. coli* H101, *Mycobacterium aurum* 4721E, *Mycobacterium phlei* ATCC 11758, and *Mycobacterium tuberculosis* strain Erdman.

Inhibitors

Tyrphostins AG 490, AG 1295, and AG 1478 and genistein were obtained from Calbiochem-Novabiochem Corp., San Diego, CA. Each was dissolved in DMSO to 5 mg/ml (tyrphostins) or 20 mg/ml (genistein). Other compounds are indicated in the text or tables. The XR series of compounds were isolated by the natural products group of Xenova Discovery Ltd. (UK).

Preparation of Culture Supernatants for Screening

Isolates were grown in 20 ml of tryptic soy broth (Difco) for a period of 3 to 4 days at 30°C with shaking. A cell-free supernatant was prepared by centrifugation at $17,000 \times g$ for 10 minutes and further clarified by passage through a 0.2 micron filter. Supernatants were stored frozen at −20°C prior to screening. Soil isolates were preserved as frozen

glycerol stocks at $-20°C$.

Characterization of Isolates

Strains of interest were characterized by fatty acid methyl ester (FAME)[15] and 16S rDNA sequence[16,17] analyses. Samples were prepared for gas chromatography according to the protocol recommended for the MIDI automated microbial identification system and were chromatographed on a Hewlett-Packard $25 m \times 0.2 mm$ phenyl methyl silicone fused capillary column on an HP 6890 system. Fatty acid profiles were compared to those in the MIDI aerobic bacteria database (MIDI, Inc., Newark, Delaware).

Primers for PCR amplification of 16S rDNA genes were GAGAGTTTGATCCTGGCTCAG (primer 16S.0007.F21) and CGGACTCCTTGTTACGACTTC (primer 16S.1491. R21) derived by J. McDermott (personal communication) from primers described by Lane[16]. PCR was carried out in a $50 \mu l$ reaction mix containing each primer at a concentration of $1 \mu M$, $200 \mu M$ of each dNTP, $20 mM$ $(NH_4)_2SO_4$, $75 mM$ Tris-HCl (pH 8.8), 0.01% Tween 20, $1.5 mM$ $MgCl_2$, 0.4 units of Ultra Therm DNA Polymerase (Bio/Can Scientific, Mississauga, Ontario), and $20 \mu l$ of template DNA prepared from isolated bacterial colonies either by heating with InstaGene matrix (Bio-Rad) or in a Fast Prep instrument (Savant) according to the supplier's protocol for Gram-positive bacteria (Bio/Can Scientific). PCR conditions included 30 cycles of denaturation (1 minute at 95°C), annealing (2 minute at 55°C), and extension (2 minutes at 72°C) in an MJ Research model PTC 100 thermocycler. Products were purified on QIAquick spin columns (Qiagen), and partial sequence was obtained on an automated sequencer using a primer with the sequence TAG(TC)GGCG(AG)ACGGGTGAGTAA (primer 16S.0099F) and ABI Prism dye terminator cycle sequencing reaction mix (Perkin Elmer). Sequences were compared by BLAST analysis[18] to available ribosomal DNA sequences.

Inhibition Assay

Sterile 6 mm filter paper discs (Schleicher and Schuell) were dipped into aqueous solutions of crude samples; approximately $25 \mu l$ saturates the disc. In the case of pure compounds, known amounts were dispensed to the discs in aliquots up to $15 \mu l$; volatile solvents were evaporated prior to application of the disc to the test culture. ISP 4 (Difco) plates were evenly spread with mycelial fragments of *Streptomyces* 85E from an overnight culture grown in tryptic soy broth and discs containing test samples were placed immediately onto these freshly seeded plates and

incubated at $30°C$[19]. Sporulation usually begins within 24 hours on this medium and plates are optimally scored for effects on development at about 36 to 48 hours after inoculation.

Inhibition studies with the other bacterial tester strains employed standard disc diffusion assays on bacterial lawns. Incubation was at 37°C for up to four weeks (in the case of *M. tuberculosis*).

Kinase Inhibition Assays

Kinase inhibition assays were carried out with kits provided by Pierce (Rockford, IL) and kinases from Upstate Biotechnology (Lake Placid, NY) were used according to the manufacturer's instructions.

Isolation and Identification of Active Compounds

General Methods

Preparative chromatography procedures were done with a Beckman HPLC instrument and system Gold software. A Hewlett-Packard HP-MSD-1100 system was used for analytical HPLC procedures. Beckman ultrapore ODS 5μ C-18, Hypersil BDS 5μ C-18 and Zorbax 5μ C-18 columns were used. An atmospheric pressure ionization-electrospray (+ve) mass detector was used for routine analysis. NMR spectra were recorded in $CDCL_3$, Cd_3OD and DMSO-d_6. ^1H NMR (400 and 500 MHz), ^{13}C NMR (125 MHz) and HMBC, NMQC spectra were obtained on a Bruker NMR spectrometer while ^{13}C NMR (75 MHz) was obtained on a Varian NMR spectrometer. Both high and low resolution FAM-MS were measured on a Kratos concept II HQ mass spectometer. Amino acid analyses were obtained with the Applied Biosystems Aminoacid Analysis model 420A/H.

Selected soil isolates identified by the assay as producers of inhibitors were grown to stationary phase in cultures up to 10 litres in tryptic soy broth (Difco). Supernatants and cell pellets were extracted separately with ethyl acetate and 10~20% methanol in ethyl acetate respectively. After concentrating both extracts and determining their activity, they were pooled and fractionated on a low mesh silica gel column, eluting with chloroform in an increasing methanol gradient.

Surfactin and Viscosin

Inhibitory activity in fractions from *Bacillus* 60A and *Pseudomonas* 11C was monitored by a thin layer chromatography bioassay method in which silica plates developed in chloroform:methanol (95:5 v/v) were transferred to square culture dishes and overlaid with ISP-4

soft agar (0.6%) containing an inoculum of $10^7 \sim 10^8$ cfu from a fresh culture of *Streptomyces* 85E. After incubation at 30°C for 24 to 30 hours, fractions with activity could be identified by inhibition of sporulation in a zone over one or more spots. Active fractions were further purified by column chromatography on Sephadex LH-20, eluting with chloroform : methanol (·1 : 1 v/v) or methanol. The active compound from *Bacillus* 60A was determined by FAB-mass spectroscopy to exhibit M^+ at m/z 1036 and high resolution FAB-MS suggested the formula $C_{53}H_{93}N_7O_{13}$. Amino acid analysis confirmed a composition consistent with surfactin. The pure compound matched an authentic sample (Sigma) by co-TLC and NMR spectroscopy.

In the case of *Pseudomonas* 11C, similar procedures yielded a pure compound with a molecular formula of $C_{54}H_{96}N_9O_{16}$, which suggested the peptide viscosin[20]. Determination of the amino acid composition and comparison with an authentic sample (a gift from Raymond Andersen) confirmed this identification.

Streptomyces Metabolites

Additional isolates selected for chemical characterization of the compounds active in the assay were identified by 16S rDNA sequence comparisons to be distinct strains of *Streptomyces*, isolated from three different soil samples.

(i) Cyclomarin A

Streptomyces D22-7B was determined to be a producer of cyclomarin A, previously identified from a marine source[21]. This heptapeptide was the compound responsible for sporulation inhibition by the crude culture broth.

(ii) Depsidomycin

Streptomyces 154M produced an active inhibitor of 85E sporulation that was isolated by organic solvent extraction. Extensive purification yielded a pure active compound with a molecular weight of m/z 791. The molecular formula of TDI-12 was determined to be $C_{38}H_{65}N_9O_9$. Comparison of spectral data with the values published for depsidomycin and amino acid analysis[22] confirmed the assignment.

(iii) Pyridomycin

Extracts of *Streptomyces* 171C had potent bactericidal activity as well as showing significant inhibition of *Streptomyces* 85E development. The crude culture broth also inhibited growth of *B. subtilis*. Extraction of a *Streptomyces* 171C culture with ethyl acetate followed by chromatography on C-18 HPLC and elution with a step gradient of methanol in water provided two UV peaks in the 80% methanol fraction; LC/MS (API-ES+ve) analysis assigned m/z 1145 $[M^+ + H]^+$ and m/z 540. The bactericidal activity was associated with m/z 1146 and sporulation inhibition with m/z 541. Subsequent structural

analysis identified these two compounds as, respectively, berninamycin A[23] and pyridomycin (erizomycin)[24].

Results

Detection of Inhibitory Activity in Bacterial Culture Supernatants

A collection of *Streptomycetes* was screened for sensitivity to known protein kinase inhibitors. A strain isolated from a wash of a lichen sample collected in British Columbia designated WEC478-85E (hereinafter strain 85E), identified as a novel *Streptomyces* by 16S ribosomal DNA sequencing, was found to be exceptionally sensitive to the effects of compounds which inhibit aerial hyphae formation (Figure 1). This strain has been deposited with the American Type Culture Collection (ATCC 55824). *Streptomyces* 85E sporulates very readily when grown on a minimal medium such as ISP4 but does not sporulate on tryptic soy agar.

The aerial hyphae assay has been employed to screen over two thousand bacterial culture supernatants for inhibitory activity. One hundred strains were found to produce compounds which prevented sporulation of *Streptomyces* 85E. Similarly, a number of known or suspected kinase inhibitors have been tested for inhibition of 85E sporulation.

Identification of Active Compounds

From the group of 100 active soil isolates, five highly active culture supernatants (large zones of sporulation inhibition) were selected for purification of the inhibitory compounds. Two of the producing strains were non-actinomycetes while the remaining three were *Streptomyces*.

Isolate 60A is a Gram-positive, spore forming rod shaped bacterium. FAME analysis identified the strain as a *Bacillus* species with a similarity index value of 0.154 to *Bacillus subtilis*. The analysis of partial 16S rDNA sequence from this strain showed greatest homology to *Bacillus licheniformis* 16S rDNA (94% identity over 400 bases). The inhibitory compound was determined to be surfactin by comparison with an authentic sample (Sigma).

Isolate 11C is a Gram-negative thin rod shaped bacterium. FAME analysis identified the strain as a *Pseudomonas* species and partial 16S rDNA sequence data showed greatest similarity to *P. putida* (98% identity over 432 bases). This strain and two other Pseudomonads produced the inhibitory compound viscosin[20]. The majority

of soil isolates in this study which scored positive as producers of sporulation inhibitors were subsequently identified as strains of *Streptomyces*. Active compounds isolated from three strains were the known cyclomarin A[21], depsidomycin[22] and pyridomycin[24].

Activity of Pure Compounds on Bacterial Growth and Development

Surfactin purified from a *Bacillus* isolate was shown to be a very effective sporulation inhibitor, with a zone of sporulation inhibition extending to 20 mm when 10 μg of the pure compound was applied to a test disc placed on a lawn of *Streptomyces* 85E. A commercial sample of surfactin (Sigma) showed very similar inhibitory activity. The inhibitory effect on sporulation was persistent, lasting for at least 3 to 4 days after the unaffected areas of the culture had sporulated (Fig. 1). Surfactin at the concentrations tested was inactive against *B. subtilis*, *S. aureus* or *E. coli* tester strains.

Viscosin, depsidomycin, pyridomycin and cyclomarin A were also identified as specific and highly effective inhibitors of *Streptomyces* 85E development in the absence of generalised bactericidal activity (Table 1). None of these natural products had been demonstrated to have activity as potential protein kinase inhibitors.

Fig. 1. The effect of streptomycete extracts on the growth of *Streptomyces* 85E.
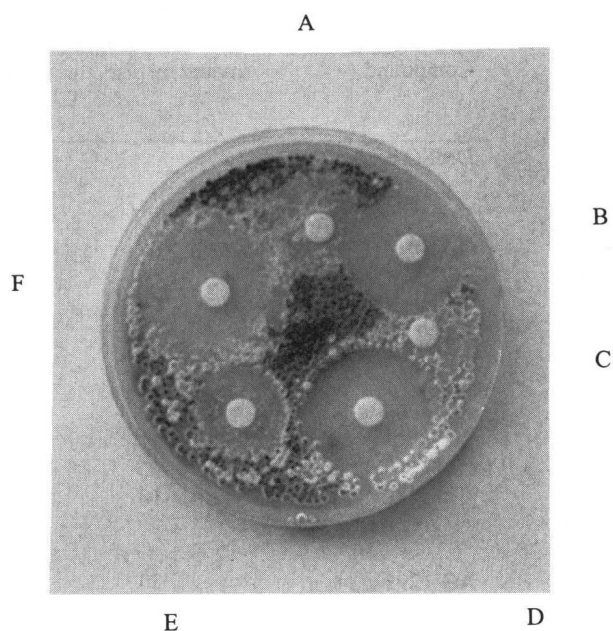
From the top in clockwise direction, the discs contain cell-free extract of strains (10 μl) that have no effect on growth or sporulation (A, C), inhibit both growth and sporulation (D), strongly inhibit aerial hyphae-formation with no inhibition of cellular growth (B:extract containing viscosin; E, F:extracts containing surfactin). The latter is typical of protein kinase inhibitors such as the tyrphostins, genistein and cyclomarin A (Tables 1 and 2).

Table 1. Effects of the bioactive compounds isolated in this study on bacterial test strains.

Soil isolate	Bioactive compound isolated in this study	Effect on sporulation of *Streptomyces* strain 85E	Effect on growth of *M. phlei* and/or *M aurum*	Effect on growth of *B. subtilis*
Bacillus strain 60A	surfactin	++	(+)	-
Pseudomonas strain 11C	viscosin	++	(+)	-
Streptomyces strain 154M	depsidomycin	++	-	-
Streptomyces strain 171C	pyridomycin	++	++	-
Streptomyces strain D22-7B	cyclomarin A	++	++	-

++: inhibition zone > 20mm   +: inhibition zone > 10mm   (+): very slight zone

-: no effect seen

The effects on development, measured as a zone of sporulation inhibition, recorded for *Streptomyces* strain 85E were produced with the application of 10 to 20 μg of each pure compound to the test disc. The effects on growth of the other strains were recorded as growth inhibition zones following application of 20 μg of each pure compound to the test disc.

Table 2. Effects of different tyrphostins, known protein tyrosine kinase inhibitors on sporulation of
Streptomyces strains.

Compound	Amount on disc, μg	Zone of inhibition of sporulation of Streptomyces 85E, mm	Zone of inhibition of sporulation of S. griseus, mm
DMSO control		0	0
AG-1478	50	30	15
	20	25	15
	10	20	11
AG-490	50	15	15
	20	0	9
	10	0	0
AG-1295	50	11	0
	20	12	0
	10	12	0
Genistein	400	14	0

Activity of Known Signal Transduction Inhibitors on Sporulation of Streptomyces Strain 85E

As controls, a number of known protein kinase inhibitors were tested for their effects on Streptomyces sporulation. Compounds such as genistein and staurosporine were found to delay the onset of sporulation for a limited time but the most striking results (Table 2) were seen with members of the tyrphostin family such as AG-1478, AG-490 and AG-1295.

The tyrphostins AG-1478 and AG-1295 were most effective; as little as 10 μg applied to a culture of Streptomyces 85E delayed the onset of sporulation. The tyrphostins were also active in inhibiting development of S. griseus; comparison of the sizes of the inhibition zones provides an example of the generally greater degree of inhibition seen when Streptomyces 85E is employed as the indicator strain.

A large culture collection of fungi and actinomycetes has been screened for inhibitory activity against isolated enzymes such as a bacterial histidine kinase[25], as well as for inhibitors of cytokine production and signalling in cell-based assays of macrophage activation[26,27] and CD28 signal transduction[28]. Several of the identified compounds were active in inhibiting sporulation of Streptomyces 85E (Table 3) with the two most active being inhibitors of macrophage activation and CD28 signal transduction. A potent inhibitor of a bacterial histidine kinase (XR587) inhibited growth of Streptomyces 85E with no discernible effect on sporulation. The two compounds most active against 85E were also capable of inhibiting growth of mycobacterial test strains (see below). XR774, the principal inhibitor found in the CD28 signalling assay, was specific in its antimycobacterial activity having no effect on B. subtilis growth, whereas XR379 also inhibited B. subtilis (Table 3).

Effects of Sporulation Inhibitors on Mycobacteria

The compounds described above were tested for inhibition of several mycobacterial strains. Mycobacteria have been shown to contain eukaryotic-like protein kinase genes[29] and it was of interest to see if inhibitors of development isolated from streptomycetes would interfere

Table 3.   Effects of novel signal transduction inhibitors isolated at Xenova Discovery Inc. on bacterial test strains.

Xenova Discovery Inc. compound	Screen where detected	Chemical description	Sporulation inhibition activity on 85E	Growth inhibition of *M. aurum* and/or *M. phlei*
XR-543	Macrophage activation inhibition	Phomalactone derivative	-	-
XR-379	Macrophage activation inhibition	Analogue of XR-543	++	++
XR-336	Macrophage activation inhibition	Resorcylic acid lactone	+	+
XR-318	Macrophage activation inhibition	Resorcylic acid lactone	+	-
XR-665	Macrophage activation inhibition	Phomalactone	+	-
XR-315	Macrophage activation inhibition	Brefeldin A	-	-
XR-475	Macrophage activation inhibition	Geldanamycin	-	-
XR-774	CD28 signal transduction inhibitor	Benzofluoranthene metabolite	++	+
XR-819	CD28 signal transduction inhibitor	Oxidised analogue of XR-774	-	-
XR-587	Histidine kinase inhibitor	Streptopyrrole	Bactericidal	++

++: inhibition seen with 20 μg/disc   +: inhibition seen with 100 μg/disc

-: no effect seen

The effects on development, measured as a zone of sporulation inhibition, recorded for *Streptomyces* strain 85E were produced with the application of 20 to 100 μg of each pure compound to the test disc. The effects on the mycobacterial strains were recorded as growth inhibition zones following application of similar quantities of each pure compound to the test disc.

with the growth of mycobacteria. The results are shown in Tables 1 and 3. It should be noted that surfactin and viscosin had only weak activity against some strains but pyridomycin and cyclomarin A were good inhibitors of *M. tuberculosis*. Cyclomarin A was previously identified as a product of a marine organism[21]; this is the first report of its isolation from a terrestrial streptomycete.

Discussion

There is increasing realization of the importance of signal transduction pathways in the maintenance of normal and aberrant cellular functions; they are potential targets for pharmaceutical intervention. There may be as many as 1000 protein kinases encoded by the human genome and together with their cognate phosphatases they present a plethora of targets for drug development for the treatment of cancer, immune, cardiovascular, neural and other diseases[11]. Natural products of microorganisms have shown potential to act as specific agents for inhibiting components of these pathways and identification of such compounds is an active area of drug discovery[30]. Similarly, the involvement of signalling processes in host infection by bacterial and fungal pathogens provides targets for novel therapies of infectious diseases[31].

Conventional assays for inhibitors of kinases and phosphatases often rely on the assessment of activity of pure enzymes *in vitro*[32]. Such assays are subject to a number of limitations and disadvantages. The purified enzymes and their substrates are often expensive and assay protocols often involve radioisotopes. The screening of microbial extracts as sources of inhibitors is often compromised by the presence of proteases and phosphatases which interfere with enzyme inhibition assays. Additionally, components of culture media are known to produce non-specific inhibitory effects. The development of a robust primary screening procedure that will detect inhibitors of protein modification directly and without interference is desirable and such a method is described here. This cell-based assay is a simple and inexpensive alternative to *in vitro* enzyme assays for the evaluation of large numbers of crude preparations from a variety of sources, including microbial and plant extracts, and combinatorial chemistry libraries. In addition, the "85E" assay system permits the ready identification of cytotoxic activity.

The "85E" assay system may be useful as a screening tool for inhibitors of a variety of signalling pathways. HONG et al.[13] first demonstrated that staurosporine and K252A, known to be inhibitors of eukaryotic protein kinases, interfered with aerial mycelium formation and sporulation of *Streptomyces griseus*. These observations were taken as evidence that protein kinases of the type found in eukaryotic cells were involved in mediation of differentiation in *S. griseus*. However, Ca^{2+} is required for aerial mycelium formation in several *Streptomyces* sp. and calcium signal modulators have also been shown to inhibit

differentiation of *Streptomyces alboniger*[33]. Verapamil and diltiazem, two well-known Ca^{2+} channel blockers, were found to be effective inhibitors of sporulation of *Streptomyces* 85E (data not shown). Hence, the bacterial screen we describe has potential in detecting novel calcium signal modulators in natural extracts or combinatorial libraries. It should be noted that the assay does not discriminate between inhibitors of protein kinases or phosphatases acting on the phosphorylated protein. We have confirmed the 85E assay by showing that known eukaryotic protein kinase inhibitors (for example, the tyrphostins) impair differentiation of *Streptomyces* (Table 2).

The variety of natural products found to inhibit sporulation of *Streptomyces* sp. further illustrates the potential of the screen for detection of a wide range of chemical moieties (Fig. 1). Development of aerial mycelia was profoundly affected by surfactin and viscosin, both lipopeptides with biosurfactant properties. Biosurfactants have the ability to partition within membranes and it is possible that a perturbation at the cell membrane acts in a non-specific manner to disrupt a signalling pathway involved in *Streptomyces* development. However, recent studies of the surfactin biosynthesis genes and mutations in these genes provide evidence that surfactin may be a regulatory molecule in *Bacillus* strains[34]. Hence, surfactin and viscosin may be acting in a specific manner to disrupt developmental signals in *Streptomyces*. TORAYA et al.[35] demonstrated that surfactin was an inhibitor of hormone activated signal transduction pathways by its ability to inhibit maturation of starfish oocytes.

The novel reduced benzofluoranthrene metabolite XR774 was a very potent inhibitor of *Streptomyces* 85E development; this compound was detected in a screen for inhibitors of CD28 induced cytokine production and was subsequently shown to inhibit selected protein tyrosine kinases including Fyn, Lck, Abl and EGF-R in *in vitro* assays with IC_{50} values in the range $20\sim400\,nM$[28]. Its oxidized derivative XR819, which is not a CD28 signal transduction inhibitor, was inactive in inhibiting sporulation, confirming the specificity of the bacterial assay. Another strong inhibitor of sporulation, XR379 was identified as an inhibitor in a macrophage activation assay and was also inhibited the *in vitro* Src assay. XR336 and XR318, two inhibitors in the bacterial test, were also detected in the screen for inhibition of macrophage activation and were subsequently shown to inhibit phosphorylation of MAP kinase[26] and also Src kinase. Taken together, these results show that *Streptomyces* 85E sporulation inhibition provides a useful approach for the

detection of protein kinase inhibitors and is not affected by inhibitors acting at other targets.

It has been found recently that a number of bacterial strains[36] possess multiple function protein kinases. Mycobacteria are among the bacterial genera that have such kinases and the completion of the genome sequence of *M. tuberculosis* led to the identification of some eleven different kinase genes[6,37]. Inhibitors of 85E sporulation were tested for their effect on mycobacterial growth. It has already been demonstrated that surfactin[38] and viscosin[20] inhibit *M. tuberculosis* and *M. avium-intracellulare* at MICs of 10~20 µg/ml with no activity against other human pathogens tested. Depsidomycin was shown to have antibacterial and immunosuppressive activity[22]. We found that pyridomycin and cyclomarin A are potent and specific *in vitro* inhibitors of mycobacterial species including *M. tuberculosis*. Compound XR774 was also active against certain mycobacterial species and some 25% of the crude extracts of streptomycete strains active in sporulation inhibition also inhibited *M. aurum*. These results suggest that there are signal transduction targets that can be used for the identification of novel classes of drugs for the treatment of tuberculosis and other mycobacterial diseases. Recent work has demonstrated another protein kinase inhibitor that is an active inhibitor of mycobacteria[41].

Regulation of secondary metabolite production and cellular differentiation in *Streptomyces* is incompletely understood at the present time. A-factor and its homologs have been implicated as autoregulatory factors of pheromone nature which exert control over signal transduction cascades leading to secondary metabolism and morphogenesis[39]. Protein phosphorylation events in bacterial signal transduction in prokaryotes with multicellular properties or in host-pathogen interactions is supported by increasing reports of the occurrence of "eukaryotic-like" protein kinase and phosphatase enzyme activities in bacteria[10,40]. It now seems probable that these enzyme activities are evolutionarily "old" and that the ancestors of eukaryotic receptor mediated signalling pathways evolved with bacterial development processes. In any event the screening strategy described here is likely to be useful for the detection of signal transduction inhibitors not readily detectable by traditional approaches, for use in the study and treatment of a wide variety of disease states in humans.

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