Inhibitors of Type-I Interleukin-1 Receptor from Microbial Metabolites

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We describe here the results of a screening program conducted to discover inhibitors of the type-I interleukin-1 receptor (IL-1RI) from samples of microbial origin. An innovative approach, based on automated, nonradioactive receptor binding assays has been employed. Specially prepared cell-free systems have allowed the use of high concentrations of microbial metabolites in the reaction mixtures with a low percentage of false positives. More than 30,000 microbial samples from different species of soil isolates have been tested and two interesting activities have been purified and characterized. One of these, isolated from *Streptomyces* sp. GE48009, was identified as niphimycin, an antifungal agent also known as scopafungin. Preliminary evidence suggests that this molecule and azalomycin F, a structural analogue, inhibit IL-1RI by virtue of their long-chain guanidinium moiety. The other activity, isolated from *Aspergillus* sp. GE49752, was identified as flavipin, a substituted *o*-phthalaldehyde.

Many harmful biological effects, such as fever, anorexia and hypotension, are known to be mediated by interleukin-1 (*i.e.*, IL-1α or IL-1β). Also, IL-1 plays a major role in pathological conditions like septic shock, inflammation and auto-immune diseases (see ref. 1, 2 for recent reviews on the IL-1 system). Consequently, there is a strong interest in the pharmaceutical industry to find effective ways to inhibit IL-1 action. *In vitro* and *in vivo* experiments with recombinant IL-1 receptor antagonist (IL-1ra) suggest that this recently discovered cytokine has a strong potential in the therapy of such pathological conditions[1,2]. On the other hand, the discovery of small, non-peptidic molecules acting like IL-1ra as specific antagonists of type-I IL-1 receptor (IL-1RI), might lead to the development of orally available, less expensive alternatives to IL-1ra. Hence, IL-1RI is currently considered as one of the most interesting targets for drug discovery.

We describe here the results obtained in the course of a high volume screening project aimed at finding IL-1RI inhibitors from natural products. We used microbial fermentation broths as sources of chemical diversity, and automated, cell-free, non-radioactive assays as high-throughput test systems. Two interesting inhibitory activities have been identified and characterized.

Materials and Methods

Microbial Samples for High Throughput Screening

Streptomycetes and fungal strains isolated from soil were inoculated in 30 ml of a culture medium having the following composition: glucose 2%, yeast extract 0.2%, soybean meal 0.8%, NaCl 0.1% and $CaCO_3$ 0.4%. Microbial cultures were grown at 28°C with shaking for 3 days (primary screening) or 4 days (secondary screening). 5 ml of each culture were centrifuged to remove mycelia and passed through 1 ml of XAD-2 resin (Fluka, Buchs, CH) to concentrate microbial metabolites. Columns were washed with water (1 ml) and then eluted with ethanol (1 ml). Eluates were dried, resuspended in 0.5 ml DMSO and adjusted to 1 ml with water. These samples were tested directly in the immobilized ligand IL-1 receptor binding assay (IL-IL1RBA) (12 μl in 60 μl final volume), or were diluted 2-fold with water (to give 25% DMSO) to be tested in the particle concentration fluorescence receptor binding assay (PCF-RBA) (20 μl in 60 μl final volume).

The 200-liter fermentation of *Streptomyces* sp. GE-48009 was carried out in the same medium used for the screening process, whereas the 200-liter fermentation of *Aspergillus* sp. GE49752 was carried out in the following medium: corn steep (5 g/liter), starch potato (10 g/liter), glucose (10 g/liter), $FeSO_4 \cdot 7H_2O$ (10 mg/liter), $MnSO_4 \cdot$

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$4H_2O$ (10 mg/liter), $CuCl_2 \cdot 2H_2O$ (0.25 mg/liter), $CaCl_2 \cdot 2H_2O$ (1 mg/liter), H_3BO_3 (0.56 mg/liter), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.2 mg/liter) and $ZnSO_4 \cdot 7H_2O$ (2 mg/liter).

Immobilized Ligand IL-1 Receptor Binding Assay (IL-IL1RBA)

The preparation of reagents and the development and validation of the assay have been described in detail elsewhere[3]. In this work, the assay was performed in a fully automated fashion, using a Beckman Biomek 1000 SL robot for all steps, from coating to spectrophotometric measurements. Briefly, flat-bottomed microplate wells were incubated with 50 μl of 3.6 μg/ml IL-1ra in PBS (50 mM NaPhosphate, pH 7.3, 150 mM NaCl) overnight, then with 250 μl/well of 3% BSA in PBS for 2 hours, washed with PBS and dried. In separate microplates, 12 μl of microbial samples or controls (50% v/v DMSO) were mixed with 48 μl of 150 pM sIL-1RI (a recombinant, soluble form of the type-I IL-1 receptor)[4] in PBSA (1% BSA in PBS). 50 μl of these mixtures were transferred to the IL-1ra-coated plates and incubated for 2 hours. Microplates were then washed twice with PBS and 50 μl of 1 : 500,000 dilution of MAb79 (an anti-sIL-1RI monoclonal antibody) ascitic fluid in PBSA were added. After 1 hour of incubation, 25 μl of 1 : 100 dilution of HRP-labelled anti-mouse IgG in PBSA were added and the incubation prolonged for one additional hour. Plates were finally washed with PBS and bound peroxidase activity was measured spectrophotometrically, using *o*-phenylenediamine (OPD) as chromogenic substrate.

Particle Concentration Fluorescence Receptor Binding Assay (PCF-RBA)

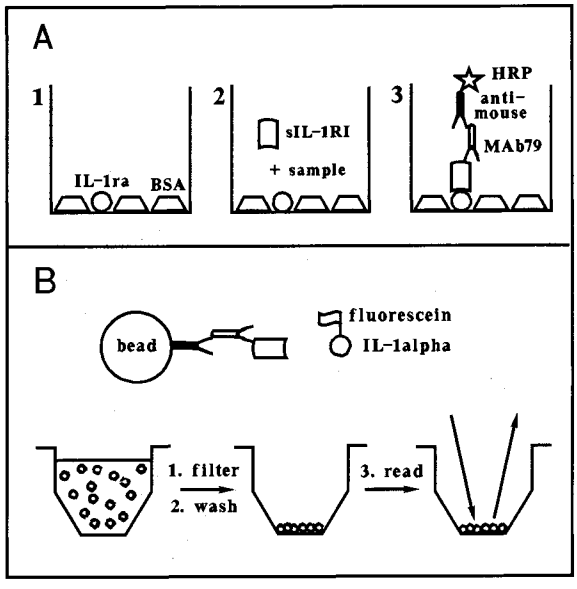
The development and validation of this assay has also been described in detail elsewhere[5]. It was performed in a semi-automated fashion, using a Screen Machine workstation (Idexx, Portland, ME). Briefly, a 0.25% w/v suspension of polystyrene particles pre-coated with goat anti-mouse IgG was mixed with an equal volume of PBSA containing 1 : 1,000 dilution of MAb79 ascitic fluid, and incubated for 2 hours at room temperature with slow agitation. The suspension was passed over a 0.2 μm cellulose acetate filter and washed with PBS. Particles were resuspended with PBSA (for background values) or PBSA containing 5 pmol/ml of sIL-1RI (to 0.25% w/v each suspension) and incubated for 2 hours with slow agitation. 20 μl of either suspension were then added to each well of Fluoricon assay plates, containing 0.22 μm filters at their bottom. Particles were filtered, washed with PBS and resuspended in 20 μl PBSA. 20 μl of sample or controls (usually 25% v/v DMSO) were added, followed by 20 μl of PBS containing 15 nM of either fluorescein-IL-1α or fluorescein-MBP-IL-1ra (a fusion protein of IL-1ra with the maltose binding protein)[5]. After 2 hours of incubation, particles were filtered, washed with PBS and drained. The amount of bound ligand was measured by exciting at 485 nm and reading the resulting epifluorescence at 535 nm.

Results

Screening of Microbial Metabolites

The high throughput primary screening was conducted with the immobilized-ligand IL-1 receptor binding assay (IL-IL1RBA), a purpose-made assay based on chromogenic detection (Fig. 1A). For the confirmation of inhibitory activities from refermented strains (secondary screening), the IL-IL1RBA was complemented by the particle concentration fluorescence receptor binding assay (PCF-RBA), based on fluorescence detection (Fig. 1B). A soluble, recombinant form of type-I IL-1RI

Fig. 1. Schemes of the IL-1RI binding assays used in this study.



(A) Immobilized-ligand IL-1RI binding assay (IL-IL1RBA):1) ligand immobilization in microtiter wells and blocking; 2) binding of sIL-1RI and 3) detection of ligand-bound receptor with primary (MAb79) and secondary antibody (HRP: horseradish peroxidase).

(B) Particle concentration fluorescence receptor binding assay (PCF-RBA): sIL-1RI, immobilized on polystyrene beads by primary and secondary antibodies, binds fluorescein-labelled IL-1α (or fluorescein-MBP-IL-1ra). Beads are filtered, washed and particle-bound fluorescence is measured. See Materials and Methods for details on both assays.

Fig. 2. Strategy and results of the high-throughput screening process for discovery of IL-1RI inhibitors from microbial metabolites.

Step	Samples	Assays *(positivity)*	Number of Samples			
			streptomyc.	rare actinos	fungi	**Total**
			10,629	11,521	10,357	32,507
primary screening	XAD elutes of ferm. broths *(72 hrs, undil., single point)*	IL-IL1RBA *(>40% inh.)*				
			63	50	38	151
secondary screening	XAD elutes of referm. broths *(72 & 96 hrs, 3 dil., duplic.)*	IL-IL1RBA *(>40% inh.)* PCF-RBA *(>40% inh.)*				
			34	11	17	62
hit characteriz.	crude extracts	IL-IL1RBA *(IC_{50}<1mg/ml)* PCF-RBA *(IC_{50}<1mg/ml)*				
			1	-	1	2

See Materials and Methods for sample preparation procedures.

(sIL-1RI) is employed in both assays, which are cell-free and very robust[3,5]. Since literature data on the IL-1RI/IL-1 interaction had led us to expect a very low hit rate for this target (see Discussion), such robustness was particularly valuable, allowing us to test fermentation samples at high concentrations, so to detect inhibitors with moderate potency or present in very low amounts. Indeed, microbial fermentation broths were routinely processed to concentrate secondary metabolites (see Materials and Methods for details), a procedure made possible by the high resistance of both assays to solvents like DMF and DMSO, used to resuspend freeze-dried samples.

During a pilot study with a small number of samples, a few light-dependent inhibitory activities were found, *i.e.*, activities that vanished when the assay was performed in the dark. Interestingly, similar findings had been previously reported by others[6] searching for inhibitors of gp120-CD4 binding, also a protein-protein interaction. Those authors found that porphyrin structures were responsible for such effect, probably as radical-forming agents in the presence of oxygen and light. To avoid this interference, we performed the entire screening process in the dark.

Fig. 2 describes the overall strategy adopted and the number of samples tested at the various stages. More than 30,000 microbial samples were tested, derived from strains isolated from soil specimens collected in different geographical areas. About 0.2% of these samples showed significant inhibition in both assays after re-fermentation (secondary screening), but almost all these inhibitory activities were not stable and disappeared during the process of crude extract preparation. Only two microbial strains, *i.e.*, *Streptomyces* sp. GE48009 and *Aspergillus* sp. GE49752, showed interesting and reproducible IL-1RI inhibition at the level of crude extracts. These inhibitory activities were purified and structurally characterized.

GE48009

The mycelium from a 200-liter fermentation of *Streptomyces* sp. GE48009 was extracted with MeOH, concentrated to water under reduced pressure and back extracted with BuOH, obtaining 338 g of dried crude material. A portion of this powder (2 g) was applied to a RP-8 column equilibrated with a mixture of aqueous $HCOONH_4$ (1 g/liter) - CH_3CN (9 : 1) and eluted with a linear gradient from 10% to 100% of CH_3CN. The active fractions (tested by IL-IL1RBA) were pooled and lyophilized, yielding 390 mg of pure material.

The physico-chemical characterization of the compound was made on the basis of IR, UV and MS spectra (see Table 1). By comparison with literature data[7], the product was identified as niphimycin (Fig. 3).

Purified niphimycin inhibits binding of IL-1ra to sIL-1RI in a dose-dependent fashion with an IC_{50} value of 100 μM (Fig. 4). A comparable activity ($IC_{50}=32\ \mu M$) was observed when the compound was tested in a radioactive, cell-based receptor binding assay using ^{125}I-IL-1β as tracer (S. YANOFSKY, personal communication). These results indicate that the inhibitory action of niphimycin is not assay- or ligand-dependent, suggesting that the compound acts directly on the receptor. Azalomycin F (Fig. 3), a niphimycin analogue[8] from our compound

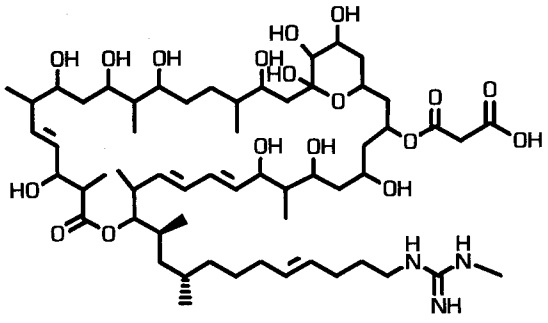
Table I. Physico-chemical properties of GE48009 and GE-49752.

	GE48009	GE49752
Appearance	Pale yellow powder	Yellow powder
Molecular formula	$C_{59}H_{103}N_3O_{18}$	$C_9H_8O_5$
Molecular weight	1143 (MH^+)[a]	197 (MH^+)[b]
UV λ_{max}^{MeOH} nm	237	209, 261, 346
IR ν_{max} (Nujol) cm^{-1}	3327, 2924, 2854, 1717, 1645, 1589, 1462, 1377	2930, 2854, 1664, 1632, 1585, 1456, 1404, 1377

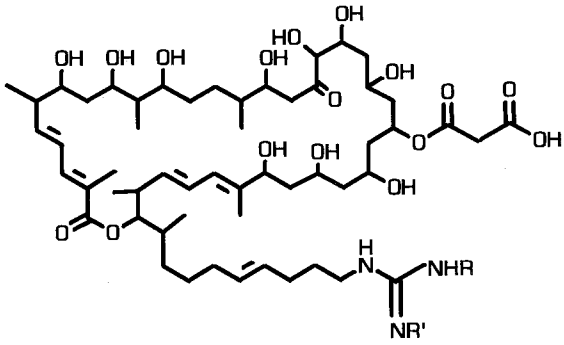
[a] FAB-MS (positive ion). The sample was dissolved in DMSO/*m*-nitrobenzyl alcohol as matrix.

[b] CI-MS (positive ion). The source temperature was 135°C and the ionization gas was methane.

Fig. 3. Chemical structures of the IL-1RI inhibitors identified in this study.

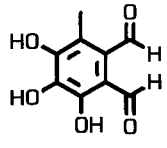


Niphimycin



Azalomycin F[a]

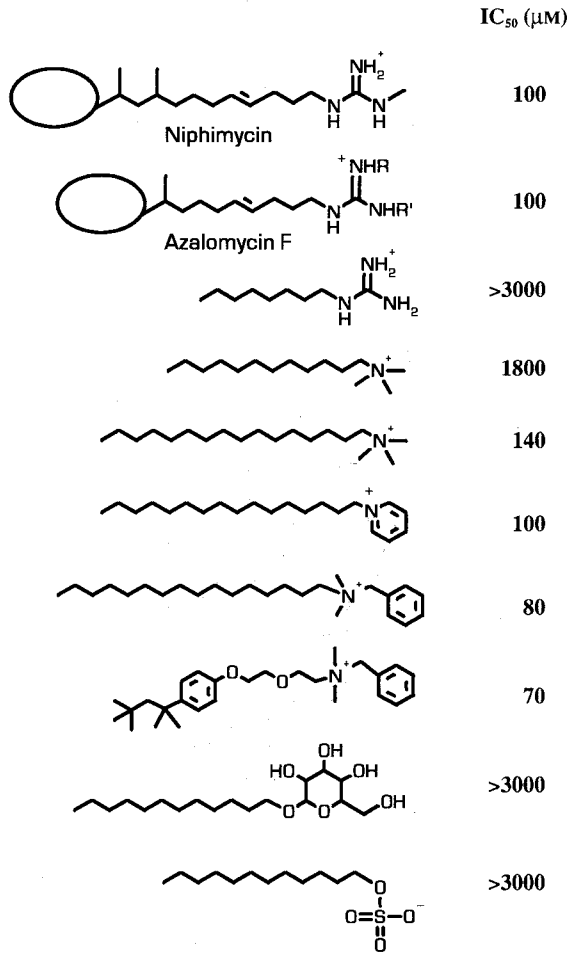
R=H or CH_3
R'=H or CH_3



Flavipin

[a] Azalomycin F is a complex of four factors differing for substituents on the guanidinium group[8].

Fig. 4. IL-1RI inhibition by different molecules, as determined by IL-IL1RBA.



The cyclic polyenic structures of niphimycin and azalomycin F are shown in detail in Fig. 3. Besides the compounds shown in the figure, the following molecules were also tested for IL-1RI inhibition: tetramethylammonium, tetrabutylammonium, guanidinium, arginine, octyl-β-D-glucopyranoside (all with IC_{50} values >3,000 μM), polyoxyethylene-9-lauryl-ether, Triton X-100 and Tween-20 (all with IC_{50} values >2% v/v).

library, inhibited IL-1RI with a similar potency (Fig. 4).

Niphimycin and azalomycin F are composed of a polyenic ring and a long hydrophobic tail terminating with a positively charged guanidinium group (Fig. 3). To assess the role of this moiety in IL-1RI inhibition, we tested a number of commercially available compounds with related structures. As shown in Fig. 4, long-chain, positively charged molecules are able to inhibit the IL-1RI/IL-1 interaction at concentrations much lower than long-chain neutral or negatively charged molecules (even when these are chaotropic agents as SDS).

GE49752

The filtered broth from a 200-liter fermentation of

Aspergillus sp. GE49752 was adsorbed on the polystyrene resin S-112, washed with distilled water and eluted with acetone - BuOH - H_2O, 8 : 1 : 1. The eluted material was concentrated to water *in vacuo* and then extracted with AcOEt. The organic layer was lyophilized yielding 9.5 g of material which was applied to a silica gel column and eluted with CH_2Cl_2 containing increasing amounts of methanol (linear gradient from 0% to 20%). The active fractions were combined, the organic solvent removed and the product lyophilized, yielding 2.11 g of material. 500 mg of this powder were further purified using the silica gel column equilibrated with Et_2O and eluted with a gradient from 0% to 50% of AcOEt - AcOH - *i*-propanol (30 : 1 : 10). The active fractions were pooled and dried under reduced pressure, yielding 250 mg of pure compound.

The structure determination was made by comparison of UV, IR and MS experimental data (listed in Table 1) with data reported in literature, leading to the identification of the compound as flavipin[9] (Fig. 3).

Purified flavipin inhibits IL-1RI in a dose-dependent fashion, both in IL-IL1RBA ($IC_{50} = 100\ \mu M$) and in PCF-RBA ($IC_{50} = 150\ \mu M$ with fluorescein-IL-1α, $IC_{50} = 250\ \mu M$ with fluorescein-MBP-IL-1ra). Like niphimycin, the inhibitory activity of flavipin is not assay- or ligand-dependent, suggesting that also this compound acts directly on the receptor. Other mycotoxins, *i.e.*, glyotoxin, citrinin, citreoviridin, chaetoglobosin A, patulin, kojic acid and nivalenol (all commercially available from Sigma) did not show any significant inhibitory activity on IL-1RI.

IL-1RI inhibition by flavipin was not affected by oxidizing agents (*e.g.*, ascorbic acid, metabisulphite) or mild reducing agents (*e.g.*, DTT), but was abolished by the presence of high concentrations (0.2 M) of lysine or arginine ($IC_{50} > 10$ mM in both cases). This finding suggests that IL-1RI might be inactivated by the reaction of flavipin with one or more of such aminoacid residues in the receptor active site.

Discussion

This report describes the results of a discovery project aimed at identifying IL-1RI antagonists from microbial metabolites. In our view, two aspects of the process are particularly interesting. The first is the ability to perform all the screening steps in a radioactive-free environment, using purpose-made assays based on chromogenic (IL-IL1RBA) or fluorescent (PCF-RBA) detection. Although unusual in the field of receptor research (^{125}I is commonly used for cytokines)[10], this feature is of great value when high throughput robotic systems are used, not only for the high price of radioactive waste disposal, but also for the containment required for personnel protection. The second important aspect of our process is the lack of cells or cell membranes in the receptor binding assays. Cell membranes limit the concentration of organic solvents (*e.g.*, DMSO, DMF) allowed in incubation buffers and, more seriously, normally yield a high number of false positives, usually membrane perturbing agents. Polyene macrolides, for instance, have been reported[11] to disrupt cell membranes by binding to cholesterol. The absence of such problems in our process is reflected in the observation that despite the high concentration of microbial metabolites in reaction mixtures, the incidence of positives was quite low (0.2%).

Niphimycin and flavipin, two molecules previously discovered for their antifungal properties, have been identified in this study as novel IL-1RI inhibitors. The activity of niphimycin and related molecules (Fig. 4) indicates that a positively charged group linked to a sufficiently long chain can effectively antagonize the IL-1RI/ligand interaction. Such an effect, not shown by neutral or negatively charged analogues, does not seem to be due to a generic detergent-like action. In this respect it is intriguing to note that, on the basis of molecular modeling analyses, it has been proposed[12] that a positively charged residue plus a hydrophobic core constituted by two solvent-exposed phenylalanine side chains provide the common binding epitope found in all three forms of IL-1 (IL-1α, IL-1β and IL-1ra). It is tempting to speculate that niphimycin and the other active molecules shown in Fig. 4 antagonize IL-1RI by mimicking such epitope.

A completely different mechanism of action must operate with flavipin. As a substituted *o*-phthalaldehyde, this small molecule is highly reactive and most likely inactivates IL-1RI by forming some kind of covalent adduct with crucial lysine and/or arginine residues. This hypothesis is supported by the scavenging action that we observed when such aminoacids were added to the reaction solution.

Despite extensive screening efforts by the pharmaceutical industry, effective small molecule agonists or antagonists for cytokine receptors have been difficult to identify. The two inhibitory activities described in this study were found in a process where more than 30,000 microbial samples were tested. Given that most microbial metabolites possess a molecular weight of less than 1,500 Da, such a low hit rate is not surprising. Indeed,

in our screening a hit was required to inhibit a high affinity binding process (K_D in the $10^{-9} \sim 10^{-10}$ M range)[2,3,5], an interaction that mutagenesis studies[12~14] suggest to involve multiple protein-protein contacts over a large three-dimensional space, and that consequently is not easily inhibited by small molecules. This observation also explains the moderate potency of the IL-1RI inhibitors described in this and other[11,15] studies (the IC_{50} values are usually in the $10^{-5} \sim 10^{-4}$ M range). On the other hand, it is important to consider that although larger structures can yield more potent inhibitors, they are also more likely to be highly immunogenic, causing undesired side-effects during the prolonged treatments required by chronic inflammatory or autoimmuno diseases. The small molecules found in this study can be interesting as tools in understanding important pharmacophores of the IL-1 receptors. In addition, niphimycin may also have a value as a model for rational design of low molecular weight antagonists with improved properties.

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