APPLICATION OF RADIORECEPTOR BINDING ANALYSIS TO THE DETECTION OF MARINE NATURAL PRODUCTS WITH THERAPEUTIC POTENTIAL

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Introduction

Historically, screening for marine natural products with therapeutic potential is most often undertaken with animate mammalian systems such as whole animals or cells grown in culture. The diversity of biologically active compounds present within marine organisms results in a very high incidence of positive results for such assays. One approach to demonstrating specificity for the extract under examination is to perform the assays in parallel on several different models. Thus, the anti-tumor screening program at the National Cancer Institute, U.S.A. employs 60 different tumour cell lines (1). Selectivity towards a sub-group of these lines provides an indication of possible applicabilty of the compounds present and controls against those extracts possessing general toxicity. In a process termed dereplication, Faulkner and coworkers (this proceedings) couples results from cytotoxicity assays, antimicrobial assays, ^1H NMR and information on related species as sources of natural products to identify organisms worthy of intense study. Similarly, Munro and coworkers (this proceedings) utilise the results from several different assays together with preliminary chromatographic properties and taxonomic information to detect potential novel biologically active compounds.

Animate assays dependent on an endpoint of decreased viability or death may be criticised on the basis that they will identify compounds whose activity may not be of particular interest e.g. a toxin with limited therapeutic potential, while not detecting compounds with regulatory rather than toxic properties.

Radioreceptor binding techniques have the potential to readily detect specific activities within natural products. Defining the term receptor broadly to include all high affinity acceptor sites, the technique may be applied to numerous drug discovery programs including agents active at ion channels and enzymes. The method involves coincubation of extracts with a receptor preparation and a suitable radiolabelled ligand. A subsequent decrease in the amount of bound radiolabel may indicate the presence of a competing ligand within the extract. Confounding results will be obtained when the extract interacts indirectly with the receptor preparation or radiolabelled ligand. Despite some potential for non-specific interactions, this technique permits the identification and assay guided purification of novel natural products with specific mechanisms.

This paper describes our practical experience in applying radioreceptor binding analysis to the identification of compounds with therapeutic potential.

Epidermal growth factor (EGF) receptor

EGF stimulates cell proliferation and differentiation *in vitro* and *in vivo* (see 2). First identified in mouse submaxillary glands (3), EGF, stimulates the the proliferation of normal precursor epithelial and fibroblastic cells (4) and wound healing (5). The effects of this class of growth factors are mediated by binding to a membrane-bound receptor. The EGF receptor exists in two affinity states, with the high affinity form mediating the EGF induced mitogenesis (6). Down-modulation of the high affinity form of the receptor is mediated by EGF and, through a process of transmodulation, other growth factors and the phorbol ester class of tumour promoters (see 7). The molecular mechanism of this processs is poorly understood. The earliest biochemical event elicited by EGF is activation of the EGF receptor's tyrosine kinase (8) leading to autophosphorylation and tyrosine phosphorylation of specific substrates (9).

Abnormalities in the EGF/EGF receptor signal transduction pathway are closely associated with the presence of some cancers. A closely related protein, transforming growth factor a (TGFa) produced by many human carcinomas appears to act as an autocrine stimulator of neoplastic cells (see 10). Also, the oncogenes, v-*erb*B and *erb*B2, encode two proteins homologous to the EGF receptor (see 11). The *erb*B2 oncogene product is overexpressed in many adenocarcinomas and nearly 30% of human breast cancer patients (see 12).

Research to develop analogues of EGF or TGFa using chemical and molecular biological techniques have been modestly successfull. Substitution of Val at position 47 of mouseEGF increases mitogenic potential but does not increase absolute affinity (6). Microorganism derived antibiotics have been identified which inhibit EGF stimulated mitogenesis (13), but their mechanism remains unclear. In addition to the utility of a novel agonist at the EGF receptor, an antagonist would have specific application in attenuating the actions of TGFa. The project was initiated as collaboration between AIMS and the Ludwig Institute to discover new tools in the regulation of mitogenic signals from this family of growth factors.

Frozen specimens collected throughout the Australasian region were sub-sampled (2-4g) and extracted in ethanol (15 ml) to provide a final concentration of ~75% ethanol. From experience, this extract provides natural products with a very broad range of chemical properties ranging from those of amino acids through to quite non-polar sterols and other low molecular weight substances. It is also amenable to direct screening using only 10ml of extract per assay tube.

Initially, screening was performed on human A431 cells grown in culture on 96-well plates. This cell line expresses large numbers of EGF receptors (≈1,000,000 /cell). To each well was added [^{125}I]EGF and 10µl aliquots of extract in triplicate. After a short incubation at 37 ^{o}C, unbound radiolabel is decanted and the cells with associated [^{125}I]EGF solubilized and counted. Non-specific binding is determined by inclusion of mg/ml concentrations of EGF. The incidence of positive results was higher than expected, with 110 of 2900 organisms producing greater than 40% inhibition of binding. To control against those agents which exert a modulatory rather than direct inhibition effect, two modified assays were employed. The first of these featured performing the incubation at 4 ^{o}C to reduce enzymic activity within the cells. The second modified assay consisted of preparing a well-washed membrane preparation from the cells which was stored frozen until use. Separation of bound and free radioligand was achieved in this assay by filtration through glass-fiber filters on which receptor bound radioactivity could then be counted. With these non-metabolizing assays, the field of potential active organisms was reduced to 15. These organisms were then tested for their activity on an unrelated radioligand binding assay ([^{125}I]growth hormone to its receptor in the rabbit liver) to test for specificity. This final stage of screening left us with a single candidate, a sponge of the genus *Crella* from the Great Barrier Reef. Within the binding assay, this extract competitively inhibited 100% of [^{125}I]EGF binding. In a model of mitogenesis which features an epidermal bowel carcinoma cell line (14) the extract while displaying considerable toxicity, also inhibited EGF stimulated mitogenesis. Thus, the organism displayed promise as a source for a new EGF receptor active compound.

The filtration assay was employed as a guide in the purification of the active component of *Crella sp.*. Reverse phase chromatography of a methanol extract of the sponge on a C18 Speedy Column (15) yielded a peak of inhibitory activity eluting between 20 and 50% methanol which was further purified on HPLC (C18, water/methanol gradient). A peak of EGF binding and antimitogenic activity was identified eluting at 30% methanol. Larger scale purifications are currently being employed to obtain sufficient material for structural elucidation.

Glutamate excitatory amino acid receptors

L-glutamate is the major excitatory neurotransmitter opening various ion channels, especially those for Ca^{2+} (16). Inappropriate overstimulation of excitatory amino acid receptors occurs in ischaemic/hypoxic episodes and has been implicated in neurodegenerative diseases including motorneurone and Alzheimer's diseases (17). The paucity of drugs available to study the glutamate/receptor interaction and the potential of an antagonist to reverse some of the degenerative processes mediated by glutamate led AIMS and the University of Melbourne to collaborate in the search for new compounds active at this site.

Marine organisms have already provided several of the pharmacological tools used to study glutamate receptors. Kainate and domoate have structural similarities to glutamate and were first identified in two red macroalgae (18,19). Subsequently, domoate was also identified as the "Amnesic Shellfish Toxin" in toxic Canadian molluscs that produced an occasionally fatal syndrome featuring neurological dysfunction (20). The source of domoate was hypothesised as a bloom of marine diatoms accumulated by the filter-feeding molluscs.

Kainate and domoate bind to a class of glutamate receptors identified as the kainate subtype. Using [^{3}H] kainate as a radioligand and a rat brain synaptosomal preparation as a source of receptors, this site may be successfully studied (21). Non-specific binding is defined by the inclusion of 50 µM glutamate. Screening of the 75% ethanol extracts of 200 organisms using this system provided us with numerous positive results (≈50%). As a means of assessing specificity, the extracts were also tested for their activity against another class of glutamate receptors labelled by [^{3}H] AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) (22). A specific inhibition of only the [^{3}H] kainate assay was observed for an extract of the Tasmanian mollusc of the genus Glycymeris, a cockle. Using assay-guided purification, it was possible to identify the active component as preferentially extracted in water. Reverse phase chromatography of this extract was performed first on C18 speedy-column (eluting at 0-10 % Ethanol) and then on semi-preparative C8 HPLC. Eluting with water containing 0.1% acetic acid provided a peak of activity of [^{3}H] kainate binding inhibition immediately after the solvent front followed by a smaller peak. Based on elution characteristics and NMR data, the active components are apparently not domoate, kainate or glutamate. We are currently attempting to further purify this activity which we hope will provide a new pharmacological tool in the study of excitatory amino acid neurotransmission.

Conclusions

The approach outlined here for the identification of marine natural products has the following advantages :

i) Specificity. By testing for activity at a discreet biochemical site at which new bioactive compounds will be of defined interest, the researcher is saved much of the guesswork associated with describing a natural products mechanism. The phenomenon of 'rediscovering the wheel' i.e. identifying a compound with little novelty with respect to mechanism is also avoided. The relative insensitivity of this method to extraneous components in the incubate which lack receptor directed activity makes it very useful with complex extracts. In particular, membrane associated receptors display a remarkable tolerance to alcohol within the assay and tolerate 5% solutions well. This feature makes it feasible to utilise alcohol containing fractions directly from reverse-phase chromatography separations.

ii) Sensitivity. Initial screening in these studies was performed on aqueous ethanolic extracts from 1-2 mg wet weight of organism per replicate providing a very economical use of collected material. Unfortunately, it is not possible to express sensitivity with respect to weight of pure compound detectable - each active compound has a characteristic potency. However, as a guide, given an extracteable abundance of 0.01% wet weight of organism (100 ng/mg), a compound with an affinity greater than 100 ng/ml will be detected readily.

iii) Rapidity. For the assays described, the total process including sample preparation, incubation and radioactivity counting time is complete within 12 hours. Membrane preparations are stable frozen for weeks so that frequent handling of the source (animals/cells) is not required.

Having stated radioreceptor binding analysis provides a specific detection system, caution should also be exercised in applying results from these studies. Misleading results may occur when the extract :

i) Illicits a change in the endogenous regulators of the receptor of interest. The receptor is subject to many factors such as ionic conditions and enzymatic modification that dramatically modulate its apparent affinity. The effect of this class of bioactive compounds may be minimised by working with non-metabolising sytems such as cell fractions or partially purified receptor preparations.

ii) Physically disrupts components of the assay. An incomplete list of such mechanisms would include agents that ; adhere to the radioligand thus making it inaccessible to the assay, or ; prevent the efficient separation of bound and free ligand e.g. by altering the properties of the filter employed.

The method we would recommend for controlling against these false positives is to utilise a similar assay in parrallel. For the EGF receptor screening we utilised an unrelated protein ligand ([^{125}I] growth hormone) binding to its receptor as a control while for the kainate binding site we also tested extracts against another receptor subtype specific ligand ([^{3}H] AMPA) thus ensuring specificity.

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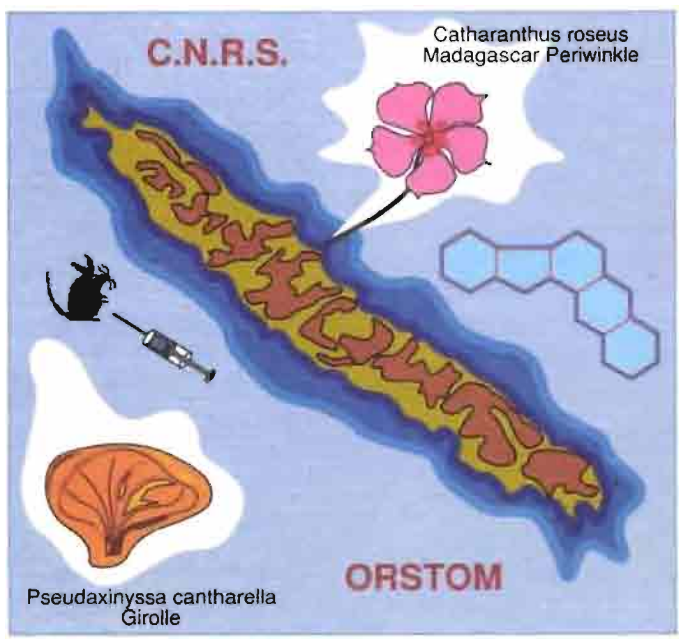

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