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SOME PHARMACOLOGICAL OBSERVATIONS ON MARINE NATURAL PRODUCTS

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INTRODUCTION

Over the last 7 years a collaborative marine chemistry and pharmacology program has evolved with the purpose of developing and extending knowledge on the pharmacological site and mechanism of action of novel marine natural products. The evolutionary process involved the development of animal models that served to detect biological activity using very small amounts of purified compounds, while also offering the opportunity to explore the mechanism of action at the tissue specific and molecular level.

A second product of this development process has also been to provide sufficient information about a particular compound to initiate interest of specialists in relevant areas of research both in academic and industrial institutions committed to drug research and development.

There have been in excess of 700 samples of marine natural products provided to us for study by Drs. John Faulkner and William Fenical, both of the Scripps Institution of Oceanography, Dr. Phillip Crews of the University of California at Santa Cruz, and Dr. Francis Schmitz of the University of Oklahoma. Our assay methods have been in some cases utilized in chemistry laboratories and in the field for the detection and eventual purification of compounds. In other cases, interesting classes of compounds have been isolated using antibiotic screening or ichthyotoxicity. Within our group there is no systematic selection procedure uniformly used by all investigators for bioassay guided fractionation—each has his own strategy and interests in marine biology. It is our perception that the pursuit of basic questions in areas such as marine biological defense mechanisms and an interest in discovering new structures of compounds often serve the goals of marine pharmacology just as well as a battery of therapeutically relevant screens.

We wish to report here that we have had measured success in identifying novel biological activity from the large pool of compounds made available to us by our collaborators. There are about 70 compounds that we are currently interested in pursuing—three compounds have reached advanced stages of study by our group. They are summarized below.

1. *Lophotoxin, a paralytic agent that binds preferentially to a nicotinic receptor subunit*

In initial testing in the rat diaphragm (*in vitro*), lophotoxin was reported to irreversibly block neuromuscular transmission and prevent nerve stimulated twitch responses. Coincident studies in mice *in vivo* showed acute toxicity at 50 mg/kg subcutaneously. The LD_{50} was subsequently shown to be 8 mg/kg.[1,2] Death was preceded by ataxia, paralysis and respiratory depression in a sequence similar to that seen with curare and α-bungarotoxin. The *in vitro* experiments were extended to studies in frog sartorius muscle for intracellular microelectrode analysis[3] and in isolated rat diaphragm to analyze the kinetics of onset of action and characterize the qualitative action in comparison to known toxins. Early in the frog sartorius muscle studies it was found that the twitch response was restored by direct muscle stimulation[3] and this was found to be also true for the rat diaphragm.[1,2]

The studies of lophotoxin reveal that the site of action of this agent appears to be exclusively postsynaptic. There is no effect on spontaneous miniature endplate potential frequency or on quantal content of evoked release.[3] Lophotoxin does not block acetylcholine (acetylcholine) muscarinic receptors but does block sympathetic ganglion transmission.[4] In receptor studies with lophotoxin, it has been found to irreversibly inactivate the nicotinic acetylcholine receptor on intact BC3H-1 cells in culture showing preference for binding on one of two sites on the nicotinic receptor.[5] Thus, this agent is highly specific for acetylcholine nicotinic receptors at the neuromuscular junction and in ganglionic synapses. Since it is irreversible, it differs from all known ganglion blockers. Thus lophotoxin is now being utilized as an important neuropharmacological probe in studying complex neuronal pathways in neurobiology. That is, the experimenter is able to selectively block out a cholinergic nicotinic pathway and assess cholinergic function in a complex set of interacting neurones. Effects studied on the mechanism of lophotoxin binding to receptors will throw light on the molecular nature of the functional units of the nicotinic receptor (Fig. 1).

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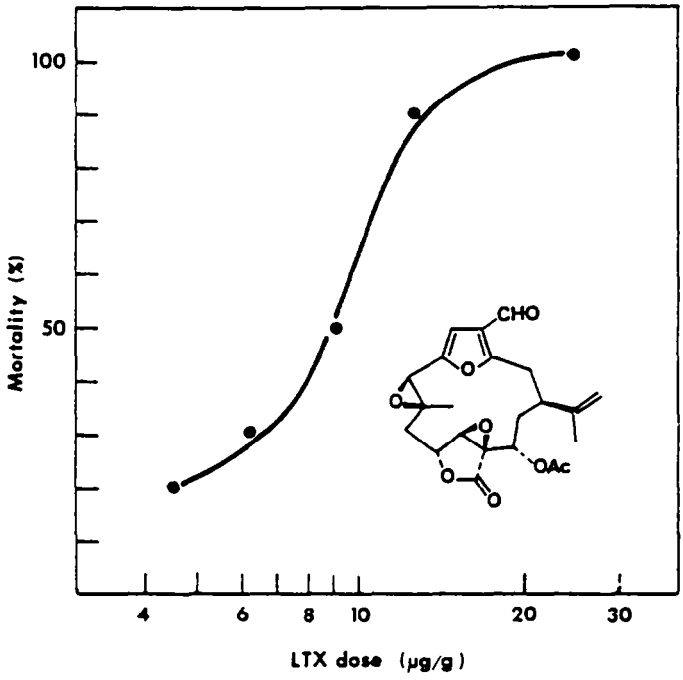

Fig. 1. Log-dose mortality curve for lophotoxin in mice. *Inset*: chemical structure of lophotoxin (from P. Culver, PhD dissertation).

2. *Manoalide*

In recent years, considerable effort has been directed toward understanding arachidonic acid metabolism and the role which this process plays in the formation of tissue autacoids that contribute to the process of pain and inflammation. A particular focus has been the study of the formation of prostaglandins following activation of phospholipase A_2 (PLA_2) and subsequent hydrolysis of cellular phospholipids. The arachidonic acid (A.A.) formed is metabolized to oxygenated products by at least two enzymatic processes: a cyclooxygenase cascade leading to prostaglandin formation and a lipoxygenase cascade leading to leukotriene formation.[6] Prostaglandins have been implicated in various forms of pain and inflammation.[7] Leukotriene formation has been implicated in immune reactions and anaphylactic shock.[8] Although considerable progress has been made in the search for compounds that block various steps in the A.A. cascade, there are at present, as far as we know, few drugs which directly inactivate PLA_2.

Manoalide is a sesterterpenoid originally isolated from the sponge *Luffariella variabilis*.[9] John Faulkner also isolated a supply of manoalide from a sponge collection obtained on the island of Palau. Manoalide was shown to be analgesic at 50 mg/kg in the phenylquinone test. The structure of manoalide is unique and unrelated to morphine, endorphin, salicylates or the indomethacin classes of drugs known to be active in this test model.

We began to explore the site and mechanism of action of this substance, on the premise that it might be a nonsteroidal anti-inflammatory agent, using phorbol myristate acetate (PMA) induced inflammation of the mouse ear.[10] This model has certain advantages for natural product work with unknown compounds. Two important ones are: (1) that the response is local involving the skin of the ear, thus the experimental model *in situ* avoids drug metabolism and excretion; (2) that this model utilizes very small amounts of drug, an important factor in the early phases of mechanism of action studies.

Positive results were found in the PMA induced inflammation assay. The potency of manoalide was greater than that of indomethacin and less than that of hydrocortisone in this assay, and histologically all three produced similar effects. Based on current theory: (1) indomethacin blocks prostaglandin formation by inhibition of cyclooxygenase; and (2) hydrocortisone blocks formation of prostaglandins by preventing arachidonic acid (A.A.) release. Manoalide, like hydrocortisone, has no effect on A.A. induced inflammation, therefore, we assumed it acted at a site prior to A.A. release and prostaglandin synthesis and by a mechanism different from indomethacin. This was further supported by time course studies showing that manoalide is most effective when applied within five minutes of PMA application, a very early point in a process which usually takes over 3 hr to fully develop.[11] In an attempt to elucidate the site of action of this compound, we examined its ability to inactivate or inhibit various actions of the enzyme PLA_2. Two studies indicated that manoalide acts directly by inactivating PLA_2. In one study, we found that it prevents the paralytic action of β-BuTX on the rat phrenic nerve-hemidiaphragm preparation.[12,13] In a biochemical study, we found manoalide prevents bee venom PLA_2 hydrolysis of phosphatidylcholine.[13,14] The binding of manoalide to PLA_2 is irreversible and pH dependent.[15] Manoalide represents a new class of pharmacological agents that are useful in exploring a wide variety of physiological processes in which PLA_2 activation could be implicated (Fig. 2).

3. *Stypoldione*

We employ the fertilized sea urchin egg as a cell culture system. Although not free of special drawbacks, this model affords a significant degree of utility

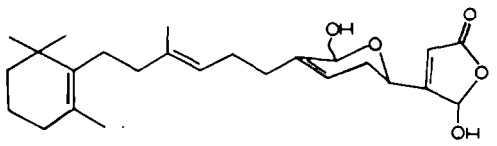

Fig. 2. Chemical structure of manoalide. (Courtesy of D. J. Faulkner.)

for investigating mechanisms of drug action as well as being a selective screen that is relatively insensitive to several classes of anti-neoplastic agents. The pharmacological advantages of this model are:

Eggs fertilized at the same time undergo synchronous divisions.

The cell cycle time is about 2 hr.

The first division of the embryo does not require mRNA synthesis because of the presence of adequate amounts of maternal mRNA.[16] Thus, when investigating drug mechanism of action, a complex set of molecular events need not be considered in the first cell cycle.

Stypoldione is an *ortho*-quinone isolated as an oxidation product of stypotriol from the brown alga *Stypopodium zonale*.[17] Pure stypoldione is lethal to fish (1 μg/ml) and was found to be a potent inhibitor of the first cleavage of fertilized sea urchin eggs.[18] In a series of studies, it was found that stypoldione inhibits microtubule polymerization *in vitro* by a novel mechanism.[19] In other studies, it has been shown that its activity is dependent on the time of addition. Stypoldione is inactive when added late in the cycle-beyond metaphase.[20] Inhibitors of cell division that are "mitotic spindle" poisons, such as the vinca alkaloids, have similar kinetics and are thought to act by blocking the ability of cells in mitosis to separate their sister chromatids at the beginning of anaphase.[19] In fixed sea urchin eggs, stained with aceto-orcein while undergoing mitosis, one detects condensed chromosomes at this stage and an absence of the nuclear membrane. Stypoldione, on the other hand, seems to present a different qualitative picture in that treated cells retain their nuclear membranes and the chromatin does not appear to be in an advanced state of condensation.[20] It appears to act on a process lasting only a few minutes and very near the onset of mitosis. In other studies of the effects of stypoldione on microtubule assembly it has been shown to bind stoichiometrically to a low affinity site on the tubulin dimer and thus reduce the rate and extent of microtubule polymerization. This mechanism of blocking polymerization contrasts with known microtubule assembly inhibitors.[19] Thus, this agent appears to have the potential of being a new pharmacological probe useful in unmasking steps in the cell cycle once its mechanism is known.

In conclusion, the study of marine natural products has provided us with the opportunity to explore and identify new classes or types of pharmacological probes. Using very restrictive screening models, we have identified three apparently unique agents. The experimental models used although not new allowed direct investigation into the putative mechanism of action of each drug, thus optimizing their eventual translation by others into a useful experimental probe or drug (Fig. 3).

EXPERIMENTAL

(a) *Rat phrenic nerve-hemidiaphragm preparations. In vitro* hemidiaphragm preparations from male Sprague-Dawley rats (150–250 g) were set up according to the method of Bülbring.[21] The physiological saline used was Krebs-Henseleit (contents in g/l: NaCl, 6.92; KCl, 0.35; $MgSO_4 \cdot 7H_2O$, 0.29; $CaCl_2$, 0.28; KH_2PO_4, 0.16; $NaHCO_3$, 2.1; glucose, 2.1) maintained at 37° and acrated with 95% O_2, 5% CO_2. Muscle contractions were evoked by administering pairs of electrical stimuli, each pair consisting of a direct stimulus applied to the muscle, followed 1 sec later by an indirect stimulus applied to the phrenic nerve. The contractions were recorded by means of an isometric transducer. The stimuli were delivered at supramaximal voltage with a frequency of 1 stimulus pair every 5 sec. Stimulus durations were 0.3–0.5 msec for the indirect, and 1–2 msec for the direct. After establishing stable control contractions, *d*-tubocurarine (*d*TC) was added at bath concentrations of $1.0–3.0 \times 10^{-6}$ M. Abolition of the indirect stimulus twitch by *d*TC with little or no effect on the direct stimulus twitch was interpreted as verification of properly applied indirect and direct stimuli. Following washout of the *d*TC and re-establishment of the control contractions, lophotoxin was added to the organ bath. In control experiments, equal volumes of the vehicle (propylene glycol) were tested in the preparation.

(b) *Microelectrode studies of the frog sartorius muscle.* All studies were performed at ambient temps using 15–30 g frogs (*Rana pipiens*). Post-synaptic potentials were observed by conventional methods (10–13) using 3 M KCl-filled micropipettes with resistances greater than 10 MΩ. Muscle contractions were prevented by including 5.0 mM $MgCl_2$ with 0.3–0.6 mM $CaCl_2$ in the bathing soln. In some experiments, 3.2–16 μM neostigmine bromide (Neo) was added to facilitate quantal analysis. Fibers were impaled under

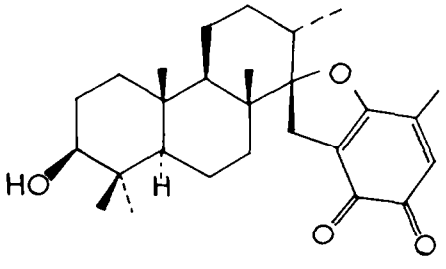

Fig. 3. Chemical structure of stypoldione. (Courtesy of W. H. Fenical.)

visual observation with audible baseline monitoring.[14] Data was only accepted from impalements which exhibited potentials with rise times less than 1 msec, and resting potentials that drifted by less than 10% during the experimental period. Potentials were recorded via a frequency modulated magnetic tape system and later analyzed on a VAS 11/780 computer. Carbamylcholine and α-bungarotoxin (α-BuTx) (from the venom of *Bungarus multicinctus*) were purchased from Sigma Chemical Co. (Saint Louis, MO, U.S.A.). LTx was kindly provided by William Fenical of the Scripps Institution of Oceanography (La Jolla, CA, U.S.A.).

(c) *Phorbol myristate acetate induced inflammation of the mouse ear.* In an adaptation of the method of Van Arman,[10] 25 microliters of an appropriate concentration of phorbol-12-myristate-13-acetate (PMA) dissolved in acetone was applied to the inner surface of the pinna of the ear and inflammation was allowed to develop for 3 hr 20 min. Immediately after sacrifice of the animal, a section through the central portion of each ear is obtained using a # 3 cork borer. The sections were then weighed and the degree of inflammation calculated from changes in weight observed in the various treatment groups.

The anti-inflammatory agents indomethacin and hydrocortisone as well as manoalide were tested by dissolving them in the PMA acetone solutions. An exception to this was the study of the time course of action of manoalide, in which separate solutions of compound were applied at appropriate intervals before and after PMA application.

Treated groups were compared with untreated controls and/or PMA control groups for statistically significant differences ($p < 0.05$) using Student's *t*-test.

(d) *Measurements of purified phospholipase A_2 activity.* Solutions of manoalide dissolved in propylene glycol were preincubated at 41° with phospholipase A_2 for 1 hr. Control tubes contained an equivalent volume of propylene glycol. 100 μl of the PLA_2-manoalide, or PLA_2-control soln, was then added to 10 ml of substrate suspension [1.36 mM phosphatidylcholine in 1 mM $CaCl_2$ and approximately 133 μl Triton X100 (pH = 7.4, temp 41°)]. The initial velocity of hydrolysis of phosphatidylcholine was measured under N_2 with a pH stat (Radiometer) as the amount of 0.005 N NaOH consumed in 1 min. The responses obtained are linear for several minutes using these concentrations of enzyme and substrate.

(e) *Cell cleavage study.* The effect of stypoldione on sea urchin embryo cleavage was determined by methods described previously.[5] Stypoldione or vehicle control was added to *Strongylocentrotus purpuratus* or *Lytechinus pictus* egg slurries at approx. 5–10 min after fertilization unless otherwise noted. Incubation temperatures for the two species were 15.5° and 18.5°, respectively. At the time of first division in controls, the percentage of stypoldione-treated embryos which had completed division was determined microscopically. Results were then expressed as percentage inhibition of egg cleavage, relative to controls. For cytological studies, 2-ml aliquots of embryos were removed at various times after fertilization and fixed in EtOH/glacial AcOH (3:1) for 8 hr, resuspended in 50% (v/v) AcOH overnight, and then stained in 2% (w/v) aceto-orcein in 50% (v/v) acetic acid for approx. 24 hr prior to examination by phase or Nomarski microscopy (X100–400).

(f) *Turbidometric assay of microtubule polymerization.* Polymerization of microtubule protein beginning with soluble protein (initiation conditions) was monitored by light scattering at 350 nm with a Gilford Model 2400 spectrophotometer using 1.4 mL curvettes with 1-cm light paths contained in a water-jacketed chamber.[10] The turbidity is directly proportional to the mass of microtubules formed and is insensitive to the lengths of the microtubules *per se*.[12] Assembly was carried out at 30° in 100 mM MES,[2] 1.0 mM EGTA, 1.0 mM $MgSO_4$, pH 6.75 (MEM buffer) at approximately 2 mg/mL total microtubule protein in the presence of 100 μM GTP and a GTP regenerating system consisting of 10 mM acetyl phosphate and 0.1 IU/mL acetate kinase.[13] The regenerating system is required for maintaining constant levels of GTP throughout the course of the experiments.

Stypoldione, dissolved in 100% ME_2SO, or ME_2SO alone, was added to the microtubule protein solution prior to initiation of assembly at 4° as 1% of the final sample volume. Samples were pipetted gently with a Pasteur pipet, then assembled by placing at 30°.

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