# IDENTIFICATION OF MACROTETROLIDE ANTIBIOTICS IN A SCREEN TO DETECT CALCIUM CHANNEL BLOCKING AGENTS

Sir:

Calcium channel blockers have been introduced into clinical medicine as a means of controlling intracellular levels of calcium. Clinical uses for these agents include the management of cardiac arrhythmias, myocardial ischemia, hypertension and hypertrophic cardiomyopathy[1]. The three calcium channel antagonists currently in clinical use are verapamil (a phenalkylamine), nifedipine (a dihydropyridine) and diltiazem (a benzothiazepine). A survey of the literature indicates that almost all known calcium channel antagonists represent structural modifications of these three compounds. These compounds form a structurally diverse group of drugs, each of which was identified as having calcium channel blocking activity on the basis of stochastic experimentation. Although all of these molecules block calcium channels, the specific properties of each structural class differ such that they offer unique therapeutic advantages for the treatment of various cardiovascular disorders. We reasoned that a new structural type, discovered by screening natural products, might possess novel properties useful both for the study of the properties of calcium channels and for the treatment of cardiovascular disease.

TAKASE *et al.*[2] had previously identified a novel calcium channel blocking agent by screening microbial fermentations using a conventional *in vitro* arterial strip assay. It seemed likely that a limitation in identifying additional calcium channel blocking compounds might be the fact that this assay is fairly slow and laborious to perform. We reasoned that the use of a simple and rapid prescreening method to sort samples for testing on arterial strips could increase the prospects for isolating new calcium channel blockers.

For this purpose, we developed a primary screen which utilized *Paramecium tetraurelia*, a single-cell organism whose swimming behavior is regulated by the activation of a voltage-dependent calcium channel[3]. Paramecium cells are completely covered with cilia which beat toward the posterior and thereby propel the organism forward. When disturbed, the cells respond with avoiding reactions in which the forward swimming is briefly interrupted by a short period of backward swimming, after which forward swimming is resumed, usually in a new direction. Backward swimming has been found to be dependent upon the opening of a calcium channel. This is clearly demonstrated in mutants, called pawns, that are incapable of backward swimming due to a genetic lesion affecting calcium channel function[4].

We hypothesized that calcium channel blocking compounds would produce the same behavioral response as a pawn mutation and that this behavioral effect could form the basis for a simple, rapid, high volume screen. An analogous behavioral assay for calcium function modulating compounds has been recently reported[5]. Preliminary experiments showed that both nitrendipine and nifedipine blocked backward swimming and elicited a "pawn" response at concentrations of 20 and 200 $\mu$M, respectively. Diltiazem did not completely block backward swimming when used alone, but when present at 20 $\mu$M decreased the concentration of nitrendipine necessary to block backward swimming to only 2 $\mu$M. Verapamil produced a variety of abnormal swimming behaviors, including circular swimming, but did not block backward swimming.

Chelating agents such as EDTA blocked backward swimming, albeit at relatively high concentrations (10 mM) and could therefore be distinguished from calcium channel blockade on the basis of potency. Several agents with various cardiovascular actions (lidocaine, procainamide, nadolol and quinidine) and a group of detergents (SDS, Tween 80, Brij 58 and deoxycholate) were also tested at a variety of concentrations and did not block backward swimming. These experiments indicated that the paramecium assay would be rapid and fairly specific although significantly lower in sensitivity when compared with the arterial strip assay. For example, nifedipine blocks backward swimming at 200 $\mu$M and shows an $IC_{50}$ of 1 nM for relaxation of KCl depolarized strips of rabbit thoracic aortae (Table 1).

We used this test to screen microbial broths in search of active leads with calcium channel blocking activity. One such lead specifically blocked backward swimming in the paramecium cells, and therefore was tested for its ability to relax KCl depolarized strips of rabbit thoracic

aortae *in vitro*. A variety of vasoactive compounds have been studied in this test system and only compounds that block calcium influx or increase cellular levels of cyclic AMP or cyclic GMP have been shown to be active at concentrations of 1 μM or less[6]. This lead relaxed the KCl contractions (demonstrated on a crude preparation before quantitation was possible), but had little effect on norepinephrine induced contraction. This finding indicates that the relaxation was not simply due to a toxic effect.

The active components of the crude preparation were isolated using the paramecium assay to guide the purification. The activity was obtained from the mycelium by first extracting with MeOH and then subjecting the extract (after removal of solvent) to a KUPCHAN extraction scheme[7]. The active $CCl_4$ fraction was further purified by high-speed counter current chromatography with a heptane - $CH_3CN$ - $H_2O$ (5 : 5 : 2) solvent system to yield several active components. The active components were subsequently identified as macrotetrolides by spectral methods ($^1H$ NMR and fast atom bombardment mass spectra (FAB-MS)) and elemental analysis. Nonactin, monactin and dinactin were present in a ratio of approximately 1 : 4 : 15. Nonactin, monactin, dinactin and trinactin are the most completely characterized members of macrotetrolide antibiotics. They have been previously isolated in various combinations and proportions from a variety of *Streptomyces* species including *Streptomyces viridochromogenes*, *Streptomyces roseochromogenes*, *Streptomyces longispori*, *Streptomyces chrysomallus*, *Streptomyces werraensis*, *Streptomyces tsusimaensis*, *Streptomyces flaveolus* and *Streptomyces aureus*[8,9]. To our knowledge, this is the first report of their isolation from *Streptomyces griseus*.

The purified macrotetrolides were subjected to pharmacological characterization *in vitro*. Potential smooth muscle relaxant activity was evaluated in isolated rabbit aortic strips, precontracted by $K^+$-induced depolarization. The potential interaction with receptors associated with the voltage-dependent $Ca^{2+}$ channel was studied by radio-ligand binding in cardiac membranes[10,11]. The results from these studies are summarized in Table 1. Nonactin, as well as monactin and dinactin induced concentration dependent relaxation of depolarized aortic strips, with $IC_{50}$ (inhibitor concentration necessary to induce 50% relaxation) values of less than 1 μM. In each case the contractile response to norepinephrine was unaltered, indicating that adrenergic contractile mechanisms of the vasculature were left intact in spite of the presence of the relaxant compound.

Nonactin, monactin and dinactin were also studied for interference with the specific binding of [$^3$H]nitrendipine to dihydropyridine receptors and of D-*cis*-[$^3$H]diltiazem to benzothiazepine receptors in purified membranes from guinea pig myocardium. The inhibition of specific nitrendipine-binding by all three compounds was nearly complete at high concentrations, whereas the corresponding inhibition of diltiazem-binding was only partial, even at the highest concentrations studied. The experimental data appear to indicate that there is good agreement between the potency values derived from relaxant activity and radio-ligand binding for monactin. Al-

Table 1. Comparison of natural products and calcium channel blockers.

| Compound | Vasorelaxant activity *in vitro* $IC_{50}$ (nM) | Radio-ligand binding | | | |
|---|---|---|---|---|---|
| | | [$^3$H]Nitrendipine | | [$^3$H]Diltiazem | |
| | | '$K_d$' (nM) | SF | '$K_d$' (nM) | SF |
| Nonactin | 115 | 8,820 | 5.34 | 15,426 | 1.06 |
| Monactin | 900 | 812 | 2.91 | 939 | 0.40 |
| Dinactin | 90 | 588 | 0.83 | 1,907 | 1.93 |
| Verapamil | 300 | ND | | ND | |
| Diltiazem | 300 | ND | | 232 | 1.05 |
| Nifedipine | 1 | 0.39 | 1.02 | ND | |
| Valinomycin | 4,250 | ND | | ND | |

'$K_d$': Apparent $K_d$.
SF: Slope factor.
ND: Not determined.

though such a correlation may suggest voltage-dependent calcium channel blockade as the mechanism of smooth muscle relaxant action, it is questionable whether the macrotetrolide antibiotics exert any significant degree of calcium entry blockade at pharmacologically relevant concentrations. It should be noted that the slope factors derived from the macrotetrolide concentration-inhibition curves, in all assays except one, deviate from 1.0 (value indicative of a competitive binding interaction). This observation and the lack of correlation between functional potency and apparent $K_d$ (binding affinity value) for nonactin and dinactin may indicate that the correlation observed is purely coincidental.

Nonactin and its derivatives have been shown to be $K^+$ ionophores and uncouplers of oxidative phosphorylation[12]. The inhibition of oxidative phosphorylation is a more plausible explanation for their activity in the paramecium and rabbit aortic tests, because the hydrolysis of ATP provides the chemical energy for ciliary movement and muscle contraction. Further support for this mode of action (uncoupling of oxidative phosphorylation) was obtained from studies with valinomycin, a cyclododecadepsipeptide antibiotic, produced by *Streptomyces fulvissimus*. Valinomycin is similar to the macrotetrolides in that it also uncouples oxidative phosphorylation[13]. Valinomycin elicited a positive response in the paramecium assay and also exhibited vasorelaxant activity in the rabbit aorta assay ($IC_{50}$ 4 $\mu$M) albeit at higher levels than the macrotetrolides tested.

In summary, the paramecium screen is capable of detecting activity of compounds known to affect voltage-dependent calcium channels. However, both the paramecium assay and the rabbit aorta test are also sensitive to inhibitors of oxidative phosphorylation. We feel that although some compounds with other modes of action are detected as positives, the paramecium assay could provide a useful primary screening tool to aid in the detection of calcium channel antagonists from natural sources.

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