Distinct binding and cellular properties of synthetic (+)- and (–)-discodermolides

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Background: Cell permeable ligands of low molecular weight can be used to dissect complex cellular processes. During the past several years this approach has been particularly important in the study of intracellular signal transduction. Discodermolide, a marine natural product, appears to inhibit a signaling pathway in immune cells. The structure of natural discodermolide is known, but its absolute stereochemistry is not. We set out to make both enantiomers and to investigate their biological activity.

Results: Both enantiomers of discodermolide were prepared by total synthesis. Surprisingly, both enantiomers have biological activity, and their effects seem to be distinct in that they arrest cells at different stages of the cell cycle. A specific binding activity was identified for (+)-discodermolide but not for (–)-discodermolide, and the binding of the two enantiomers was not competitive.

Conclusions: Both enantiomers of discodermolide have antiproliferative activity, but they act by distinct mechanisms and appear to have distinct cellular targets. The natural product is the (+)-enantiomer, which blocks the cell cycle in the G2 or M phase. The (–)-enantiomer blocks cells in S phase. Both may be useful in studies of the regulation of the cell cycle; we have also identified a specific binding activity for (+)-discodermolide, and have provided evidence that it interacts with a functionally relevant receptor.

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Introduction

Lipophilic natural products can be used to study intracellular processes in a way that is analogous to the use of genetics [1,2]. Whereas the genetic approach uses gene modification and complementation to identify proteins that are essential for a particular pathway or activity, the chemical approach uses a specific binding event to identify targets that are essential for a given function. In the case of signal transduction, both approaches can lead to the direct discovery of signaling proteins, and they can also indirectly reveal downstream components of the pathway. A promising candidate for this type of approach is the marine natural product discodermolide [3], isolated from the sponge *Discodermia dissoluta*. This compound inhibits T-cell proliferation with an IC_{50} value of 9 nM and exhibits immunosuppressive activity in mice [4,5].

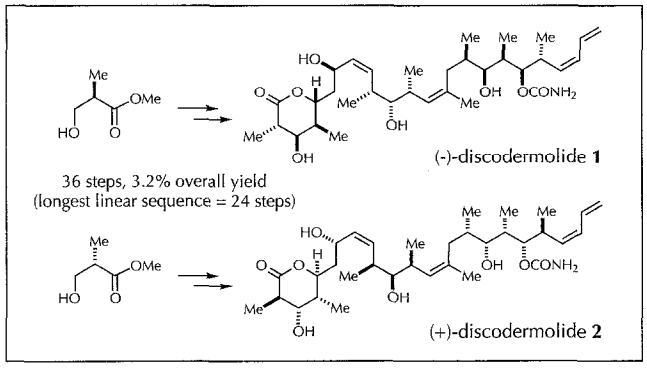

Fig. 1. Structures of (–)- and (+)-discodermolide.

Only small quantities of the compound could be isolated from the natural source; we thus undertook a total synthesis of discodermolide in order to obtain quantities that would allow a detailed investigation of its cellular mechanism of growth inhibition. Our initial synthesis [6] produced (–)-discodermolide; although the optical rotation of this compound was opposite in sign from that reported for natural discodermolide, the values were small enough to cast doubt on the use of these data as the sole basis for stereochemical assignment. Given this ambiguity, we decided to synthesize both enantiomers (Fig. 1).

For maximum efficiency we chose a convergent approach to the synthesis, dividing the molecule into three fragments of approximately equal complexity that would be coupled relatively late (Fig. 2). The molecule contains symmetry elements with a common stereochemical triad that could be generated using two homoallylic alcohols (compounds 7 and 8, Fig. 2), both prepared from a common methyl ester by a procedure reported by Roush and coworkers [7]. The (+)-enantiomer (compound 2, Fig. 1) was readily synthesized in a manner analogous to that reported for (–)-discodermolide (compound 1) [6], using methyl (*S*)-(+)-3-hydroxy-2-methylpropionate as the starting material rather than the *R* enantiomer.

We characterized the biological activity of the two enantiomers by several methods. Initially, we hoped to determine the absolute stereochemistry of the natural product by determining which of the synthetic compounds, with known stereochemistry, had antiproliferative activity. Surprisingly, both enantiomers inhibited the

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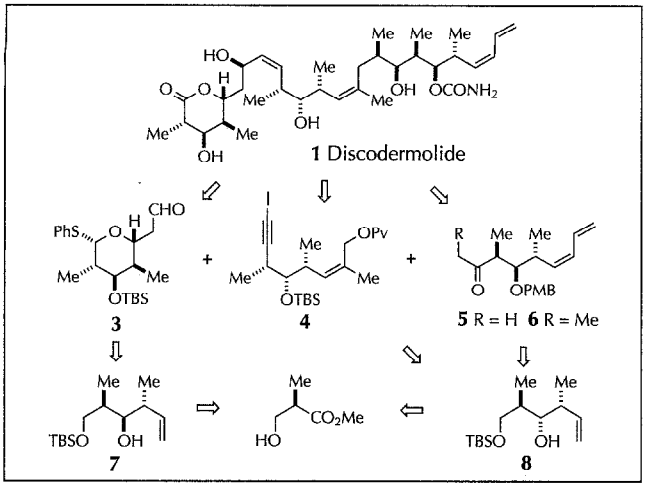


Fig. 2. Retrosynthetic analysis of discodermolide.

incorporation of tritiated thymidine in several cell lines. We therefore characterized the mechanisms of inhibition of the two discodermolides further, using flow cytometric analysis. The cycle in which cells divide and grow has been divided into four defined phases, G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis), which can be distinguished by flow cytometry, as cells in the different phases have different DNA content. The two compounds arrested growth at different stages of the cell cycle. Finally, to determine whether the discodermolides mediate their effects through distinct cellular targets, tritiated samples of the discodermolides were synthesized and used to identify specific cellular binding activities. We also analyzed the ability of one enantiomer to bind in competition with the other. The results of these experiments indicate that the two compounds bind to different targets, and therefore inhibit cell cycle progression by mechanisms that seem to be unrelated.

Results and discussion

The (+)-enantiomer (see Fig. 1) was synthesized from methyl (*S*)-(+)-3-hydroxy-2-methylpropionate by the method of Nerenberg *et al.* [6]. Spectral data for all intermediates and the final product were consistent with their respective enantiomers; the optical rotation for the final product was determined to be $[\alpha]_D^{20} = +14.0$ (c = 0.6, methanol), and for (–)-discodermolide the value is $[\alpha]_D^{20} = -13.0$ (c = 0.6, methanol) [5]. Natural discodermolide has been reported to have a rotation of $[\alpha]_D^{20} = +7.2$ (c = 0.72, methanol) [3].

Table 1. IC_{50} values for (+)- and (–)-discodermolide in a ^{3}H-thymidine incorporation assay for different mammalian cell lines.

	NIH3T3	MG63	Jurkat
(–)-Discodermolide	135 nM	72 nM	480 nM
(+)-Discodermolide	7 nM	6 nM	83 nM

To learn more about the properties of the two enantiomeric forms, we compared their effects on several cell lines. Longley *et. al.* have reported that the natural product suppresses the proliferation of purified T cells with an IC_{50} value of 9.0 nM [4,5]. The activities of the two synthetic enantiomers were first assayed by measuring their ability to inhibit the incorporation of exogenous tritiated thymidine (^{3}H-thymidine) into the DNA of proliferating cells. Three different cell types, human transformed T cells (Jurkat), mouse fibroblasts (NIH3T3), and human osteoblasts (MG63), were treated with varying concentrations of the two discodermolides. We were surprised to find that both enantiomers inhibit ^{3}H-thymidine incorporation

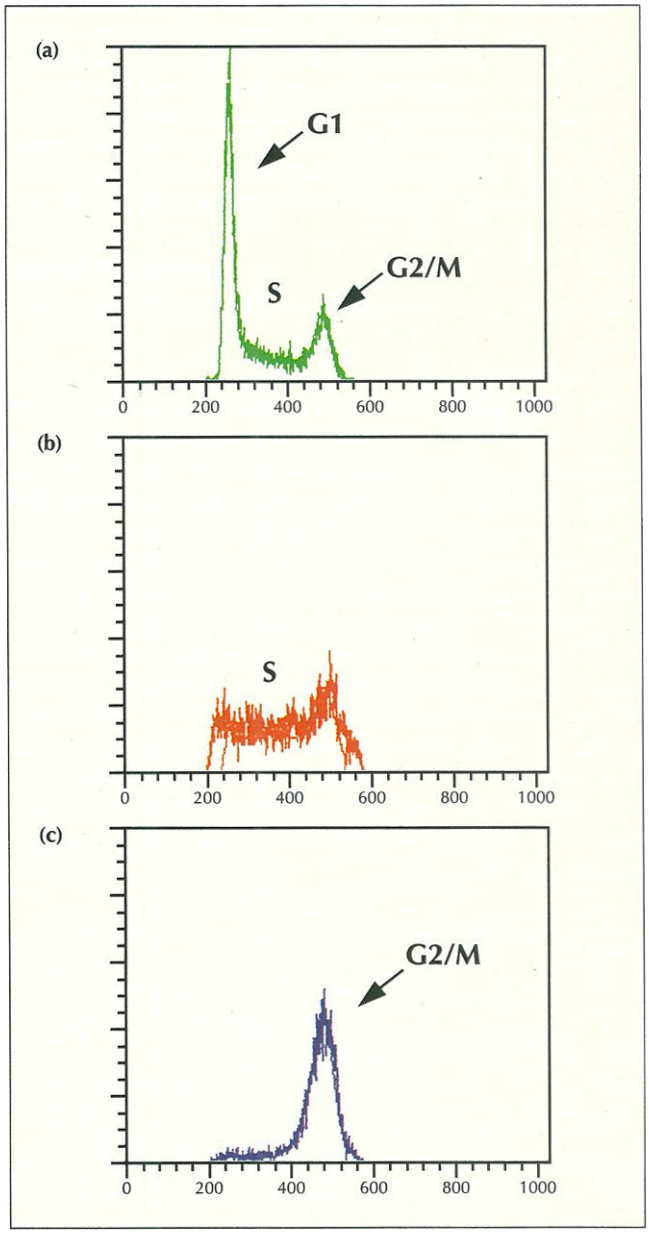
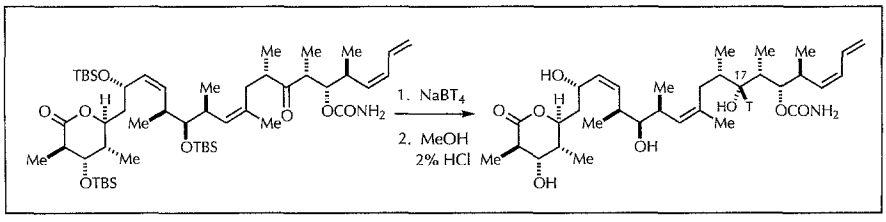


Fig. 3. Flow cytometric analyses of MG63 cells treated with synthetic (–)- and (+)-discodermolide. **(a)** Asynchronous population of cells. **(b)** Cells treated for 36 h with 1 μm (–)-discodermolide. **(c)** Cells treated for 36 h with 1 μm (+)-discodermolide. The ordinate of the graphs plots cell number and the abscissa plots fluorescence intensity.

Fig. 4. Tritiation and deprotection of a late discodermolide intermediate.



(Table 1). In addition, their antiproliferative effects were observed in all three cell lines (Table 1), thus demonstrating that discodermolide's actions are not limited to cells of the immune system [4,5].

In ^{3}H-thymidine incorporation assays (Table 1), (+)-discodermolide had IC_{50} values of 7 nM and 6 nM in NIH3T3 and MG63 cells, respectively, compared with 135 nM and 72 nM for (–)-discodermolide. Although the (+)-enantiomer was consistently on the order of ten times more potent than the (–)-enantiomer, the potency of (–)-discodermolide was nevertheless significant. At 1 μM, there appeared to be no cytotoxicity for the (–)-enantiomer and only minimal cytotoxicity for the (+)-enantiomer, as determined by trypan blue dye exclusion [8].

In light of the small rotation measured for the natural product (see above) and our inability to acquire a natural sample for direct comparison, we were hesitant at first to assign the absolute stereochemistry of natural discodermolide. However, the combined information gained from side-by-side measurements of optical rotation for the synthetic enantiomers and the comparison of the antiproliferative activities of the synthetic products with that reported for natural discodermolide (IC_{50} = 9 nM) now allows us to assign the natural stereochemistry as that drawn for the (+)-enantiomer (compound 2) in Fig. 1.

The surprising finding that both enantiomers inhibit the proliferation of cells led us to undertake a preliminary mechanistic investigation. The synthetic compounds were analyzed by flow cytometry [9,10] to determine whether they cause cells to undergo cell cycle arrest, and if so, to determine the nature of the arrest. This type of analysis relies on the fact that propidium iodide (PI) fluoresces when bound to nucleic acids. The fluorescence intensity of individual, PI-stained cells is proportional to their DNA content. Actively proliferating cells undergo a cycle from G1 phase (when they have a normal DNA complement, 2N), through S or synthesis phase, during which they double their DNA content, to G2 phase (when they have completed DNA synthesis and have twice as much DNA as a cell in G1, i.e. 4N), and finally to mitosis or cell division which returns them to a G1 state. By measuring the DNA content within cells, the distribution of a population of cells in the cell cycle can be determined statistically.

MG63 cells were treated with 1 μM of (–)- or (+)-discodermolide for 36 h (cycling time ≈ 24 h) and then subjected to flow cytometric analysis (Fig. 3). An asynchronous, untreated population of cells is mainly in the G1 phase (Fig. 3a). By comparison, a population treated with (–)-discodermolide contained significantly more cells in S phase, as demonstrated by the increased number of cells with DNA content between 2N and 4N. In contrast, cells treated with (+)-discodermolide were arrested at the G2 or M phase. The finding that the two enantiomers arrest cells at different phases of the cell cycle indicates that they have distinct mechanisms of action. In addition, it has been reported that the actions of natural discodermolide are reversible [11], indicating that the compound may be useful in synchronizing cells.

To determine whether there might be specific cellular receptors for the discodermolides, we synthesized radiolabeled variants of the two enantiomers (Fig. 4). To detect specific binding activities, MG63 cells were treated with 100 nM ^{3}H-(+)- and (–)-discodermolides for 26–36 h, harvested, and lysed. The cellular proteins were then precipitated with polyethylene glycol (PEG) in the presence of γ-globulin and centrifuged to separate proteins (and thus discodermolide bound to protein) from free, unbound discodermolide [12]. Bound, tritiated discodermolide was quantitated by scintillation counting. Nonspecific binding was detected by pretreatment of the cells with a 10-fold excess of cold, unlabeled discodermolide, followed by the addition of the appropriate ^{3}H-discodermolide. (+)-Discodermolide clearly exhibited specific binding; however, under these same experimental

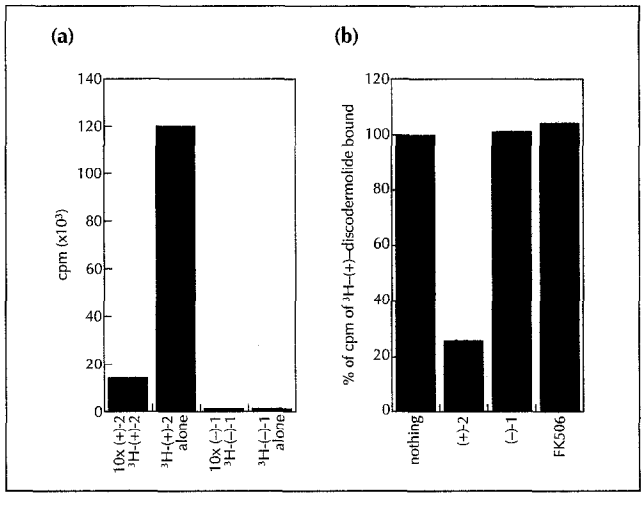


Fig. 5. (–)-Discodermolide does not compete with (+)-discodermolide for binding. **(a)** Plot showing counts per minute (cpm) of ^{3}H-(+)- or (–)-discodermolide bound in MG63 cell extracts after treatment alone (100 nm) or with 10-fold excess of unlabeled compound (1 μm). **(b)** Plot showing the percentage of cpm of ^{3}H-(+)-discodermolide (100 nm) still bound after exchange with 10-fold excess of various compounds (1 μm).

conditions, no specific binding was detected for (–)-discodermolide, even at concentrations up to 1 μM (Fig. 5a). This result does not, however, rule out the existence of a specific receptor for (–)-discodermolide that may have escaped detection because of its low abundance.

To determine whether the two enantiomers bind competitively, MG63 cells were arrested in G2 or M phase with 100 nM ^{3}H-(+)-discodermolide for 26–36 h, and then incubated with a 10-fold excess of cold (+)- or (–)-discodermolide to allow exchange of the labeled compound. Cold (+)-discodermolide showed, as expected, significant competition for its target protein (Fig. 5b). In contrast, 1 μM of FK506 [13] (an unrelated structure), and (–)-discodermolide did not compete for ^{3}H-(+)-discodermolide binding. These results suggest that the two synthetic enantiomers arrest cells at different phases of the cell cycle by interacting with distinct targets. Whereas the natural (+)-discodermolide has a specific protein-binding activity, (–)-discodermolide may either interact with a low-abundance or low-affinity protein receptor, or bind to a non-protein site.

We have also synthesized several structural variants of the natural compound and used them as probes to correlate binding with cellular function. One of these compounds, 17-*epi*-hydroxy-discodermolide, which results from deprotection of the minor (17*S*)-alcohol produced in the sodium borohydride reduction of the precursor ketone (Fig. 4), was analyzed in the same assays as the natural epimers. This non-natural diastereomer was completely inactive in the tritiated thymidine incorporation assay at concentrations up to 10 μM, and (+)-17-*epi*-hydroxy-discodermolide could not compete with ^{3}H-(+)-discodermolide in the binding assay (data not shown). Thus, binding to the putative receptor for (+)-discodermolide correlates well with the ability to inhibit cell proliferation, indicating that the receptor is both specific for (+)-discodermolide and functionally relevant.

Significance

An understanding of the exquisite regulation of the cell cycle may be crucial to dissect the mechanisms governing normal processes such as cell growth, as well as abnormal ones such as cancer. Significant progress has been made in identifying various elements of this regulation, particularly several families of protein kinases and phosphatases which activate and inactivate each other, but much remains to be done [14,15]. Natural products that inhibit proliferation by causing a specific block in the cell cycle are currently a relatively untapped resource, which may provide tools to examine cell cycle control. Discodermolide is one example of this class of compounds.

In the course of attempting to produce quantities of synthetic discodermolide sufficient for its activity to be studied, we synthesized both possible enantiomers of the compound. Surprisingly, both have activity in an antiproliferative assay, and the two enantiomers appear to have different effects and bind to different targets. The (+)-enantiomer arrests cells in the G2 or M phase, and appears to have a specific protein receptor. The use of labeled (+)-discodermolide may thus lead directly to the identification of a molecule involved in these stages of the cell cycle. The target for the (–)-enantiomer is less well defined, but since this compound causes arrest in S phase it may also provide insight into the control mechanisms that operate in this phase of the cell cycle. In that our original intention was to develop a probe for the study of cellular mechanisms, the synthetic efforts on discodermolide have returned double what was originally anticipated.

Materials and methods

(+)- and (–)-Discodermolide

(+)- and (–)-Discodermolides were obtained by total synthesis by the method of Nerenberg *et al.* [6] starting from *S*-(+)- and *R*-(–)-3-hydroxy-2-methylpropionate, respectively. All intermediate compounds were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), infrared spectroscopy (IR), high-resolution mass spectrometry and optical rotation.

Tritiated thymidine incorporation assay

For maintenance of cell lines, MG63 human osteosarcoma cells and Jurkat human lymphoma cells were grown in RPMI 1640 and 10 % fetal bovine serum (FBS) at 37 °C and 5 % CO_2. The NIH3T3 mouse fibroblast cell line (ATCC, Rockville, MD) was maintained in DMEM and 10 % FBS.

In 96 well plates, approximately 1x10^4 cells were seeded in each well in 200 μl media. After 12 h, (+)- or (–)-discodermolide in 20 % ethanol was added to obtain final concentrations that ranged from 0 μM to 100 μM. All samples were done in triplicate. After waiting 36 h for complete arrest, cells were pulsed with 1 μC_i ^{3}H-thymidine (6.7 C_i mmol^{-1}, New England Nuclear) for 6 h. For adherent cells, NIH3T3 and MG63, the media was removed; the cells were trypsinized with 50 μl trypsin-EDTA, and 150 μl fresh media were added to terminate trypsinization. For Jurkat cells, which grow in suspension, the cells were used directly. Cells were harvested by a semiautomatic cell harvester (Skatron), which transferred cellular DNA to glass fibre filters. The filters were individually excised and counted in a scintillation counter. Lineweaver–Burke analyses were performed to determine K_i values.

Flow cytometric analysis of cell cycle arrest

Analysis was done on both synchronous and asynchronous MG63 cell populations. To synchronize cells, 8x10^5 cells were plated in a 75 cm^2 flask and allowed to adhere overnight. Cells were washed twice with serum-free RPMI medium, then serum-deprived for 48 h in RPMI/0.4 % FBS. Cycling was restored by exchanging this medium for complete medium with 10 % FBS.

Cells were arrested by treating either asynchronous cells or synchronous cells at the time of release with 1 μM (+)- or (–)-discodermolide for 36 h. Cells were then trypsinized, washed twice with Hanks' balanced salt solution (HBSS), and fixed in 250 μl HBSS

and 750 µl 95 % ethanol. After incubating for 30 min on ice, cells were pelleted at 1 000 rpm and resuspended in 250 µl RNase (1 mg ml^{-1} in PBS). After 30 min at 37 °C, cells were pelleted and resuspended in 1 ml propidium iodide (50 mg ml^{-1} in 0.1 % sodium citrate). After 30 min at 4 °C, cells were analyzed on a Becton Dickinson FACS-Star instrument.

Tritiation and deprotection of discodermolide

A late intermediate in the synthesis by Nerenberg *et al.* [6] and its antipode (3 mg (3.2 µmol) of 17-keto-tri-*tert*-butyldimethyl silyl discodermolide in 50 µl THF) were reduced with $NaBT_4$ (35 µmol, 70 Ci $mmol^{-1}$, New England Nuclear) for 4 h at room temperature. The reaction was quenched with a drop of methanol, concentrated *in vacuo*, and chromatographed on a SiO_2 column (20 % ethyl acetate/hexane) to separate the major and minor alcohols, which are epimeric at C_{17}.

The major alcohol was dissolved in 200 µl of a 5 % HCl in methanol solution and stirred at room temperature for 12 h. The reaction was quenched by the addition of solid $NaHCO_3$ and chromatographed (5 % methanol/chloroform) to provide a sample of tritiated discodermolide in 25 % yield for two steps. The specific activity was calculated to be 8 C_i $mmol^{-1}$.

Specific binding activity assay

In 6-well plates, $1x10^5$ MG63 cells in 2 ml were seeded per well. Cells were allowed to adhere overnight before the addition of discodermolide samples. For measurement of total binding, 100 nM ^{3}H-discodermolide was added for 28 h. For measurement of nonspecific binding, 1 µM unlabeled discodermolide was added for 26 h, followed by the addition of 100 nM ^{3}H-discodermolide for 2 h. Specific binding is the difference between total and nonspecific binding.

Cells were harvested by adding 500 µl trypsin-EDTA, centrifuging at 1 000 rpm for 10 min, washing once with 1 ml PBS, and centrifuging again. Cells were lysed by the addition of 500 µl Triton X-100 lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1 % Triton X-100, 1 mM DTT, protease inhibitors) and incubation on ice for 5 min. To precipitate the soluble proteins, γ-globulin was added to a final concentration of 1 mg l^{-1}, followed by the addition of 500 µl 25 % solution of polyethylene glycol MW 8 000 (to a final concentration of 12.5 %). After incubation on ice for 10 min, precipitated proteins were pelleted at 12 000g for 5 min. The pellet was resuspended in a 10 % SDS solution and counted in a liquid scintillation counter.

Controls were done to show that less than 1 % of free ^{3}H-discodermolide is precipitated and pelleted under these conditions and that more than 95 % of soluble proteins are precipitated. The counts measured for the various samples were standardized by protein concentrations, which were measured by a Bradford assay (Biorad).

Enantiomer competition assay

$1x10^5$ MG63 cells in 2 ml were plated in wells of 6 well plates. After adhering, cells were arrested with 100 nM ^{3}H-(+)-discodermolide for 26 h, then 1 µM of (+)-discodermolide, (–)-discodermolide, or FK506 was added for 6 h to allow exchange. Cells were harvested, lysed, and analyzed by the method described above for the specific binding assay. Again, counts were standardized according to their protein concentration and expressed as a percentage of activity that remained bound to protein. The control, containing no added competing compound, was defined as 100 %.

This method of exchange, pretreatment and thus arrest with the ^{3}H-(+)-enantiomer followed by addition of the cold competitor, was performed in order to remove any variation in binding of the ^{3}H-(+)-enantiomer observed when cells are arrested at different stages in the cell cycle. We observed that cells arrested in the G2 or M phase tended to have 5-fold higher binding than at any other stage in the cell cycle. Thus, treatment of cells with the two different enantiomers at 10-fold excess before arrest at the G2/M phase should lead to anomalous results since addition of the (–)-enantiomer would have resulted in some S phase-arrested cells.

At 10 µM, exchange of (–)-discodermolide for the ^{3}H-(+)-enantiomer was observed; however, since no exchange was observed at 1 µM, it is likely that this is artefactual, rather than the result of true competition.

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