A Phenotypic Screening Approach to Identify Anticancer Compounds Derived from Marine Fungi

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ABSTRACT

This study covers the isolation, testing, and identification of natural products with anticancer properties. Secondary metabolites were isolated from fungal strains originating from a variety of marine habitats. Strain culture protocols were optimized with respect to growth media composition and fermentation conditions. From these producers, isolated compounds were screened for their effect on the viability and proliferation of a subset of the NCI60 panel of cancer cell lines. Active compounds of interest were identified and selected for detailed assessments and structural elucidation using nuclear magnetic resonance. This revealed the majority of fungal-derived compounds represented known anticancer chemotypes, confirming the integrity of the process and the ability to identify suitable compounds. Examination of effects of selected compounds on cancer-associated cell signaling pathways used phospho flow cytometry in combination with 3D fluorescent cell barcoding. In parallel, the study addressed the logistical aspects of maintaining multiple cancer cell lines in culture simultaneously. A potential solution involving microbead-based cell culture was investigated (BioLevigator, Hamilton). Selected cell lines were cultured in microbead and 2D methods and cell viability tests showed comparable compound inhibition in both methods ($R^2 = 0.95$). In a further technology assessment, an image-based assay system was investigated for its utility as a possible complement to ATP-based detection for quantifying cell growth and viability in a label-free manner.

INTRODUCTION

The urgent need for novel substances for the treatment of severe human diseases such as cancer, combined with the recognition that marine organisms provide a rich potential source of such substances, support the intensive exploration

of new substances from marine organisms. To date, some 20,000 natural products from marine organisms have been identified since investigation of this resource began in the 1970s.¹ Half of the published articles on marine natural products (MNP) report bioactivity data for new compounds.² Nevertheless, there are only seven drugs on the market based on MNPs, showing a considerable gap in the translational process.^{3,4} Major bottlenecks include difficulties in the reproduction and the scale up of production processes to generate sufficient compound quantities for further testing in clinical and preclinical phases.⁵ Today, there are more options for improving their production yields using modern fermentation technologies and strain development strategies. The potential of new MNPs is considerable given that multiple bioactive secondary metabolites can be produced by a single species.⁶ About 800 species of obligate marine fungi have been reported.^{7,8} Identification of anticancer compounds represents a promising area of investigation given that 10 of the 11 MNPs in clinical trials are targeting cancer.³

Demonstration of anticancer activity of a given compound can involve a range of assays monitoring proliferation, viability, apoptosis, angiogenesis, and antitumor immunity, as well as target-specific effects, signal pathways, and biomarkers. In this study, we used a subset of the National Cancer Institute (NCI) panel of 60 cell lines, which represent a wide variety of cancer phenotypes.⁹ By assessing cell viability before compound exposure, it is possible to differentiate cytostatic and cytotoxic effects. The screening procedure of the Developmental Therapeutics Program of the NCI and National Institutes of Health (NIH) is based on a colorimetric protein quantification assay using sulforhodamine-B.^{10,11} Other common formats use adenosine triphosphate (ATP) detection in luminescence-based assays (described in *Supplementary Table S1*; *Supplementary Data* are available online at www.liebertpub.com/adt) as a surrogate readout for cell number, and can routinely be conducted with <500 cells per well—a relatively low number compared to fluorescence- or colorimetric-based methods, which can require tens of thousands of cells per well. ATP detection requires the lysis of the cells followed by addition of appropriate detection reagents containing luciferase and luciferin, as well as a variety of proprietary buffer components, which serve to produce a stable luminescence signal lasting up to 2 h.¹² Nondestructive testing of cell viability and proliferation using label-free optical readouts offers the ability to perform kinetic assays, as well as potential reductions in costs and time compared to conventional formats. In this study, we employed ATP detection (Promega CellTiter-Glo) for routine profiling of multiple cancer cell lines. For a limited number of cell lines, we also compared this technique to a label-free readout (Cell Metric, Solentim Ltd.)¹³ of cell viability and proliferation.

The phospho flow technique can provide a guideline for downstream target deconvolution and mode-of-action studies.¹⁴ Cells are stimulated in the absence or presence of test compounds, and intracellular phosphorylation events are then analyzed using phospho-specific antibodies. Fluorescent cell barcoding (FCB) provides further information, as it enables combinations of up to 48 samples from different conditions to be assessed within a single experiment.^{15,16} Following the phosphorylation signals from membrane-bound receptors down to late signaling events such as phosphorylation of transcription factors, it can be directly shown if and—depending on the number of phospho epitopes analyzed—where a compound interferes with a certain signaling pathway. The phospho flow tool thereby is able to provide a starting point for further specific in-depth analyses to reveal a compound's target(s).

Profiling of compounds against multiple cell lines in parallel imposes a significant practical burden in terms of the complexity and scope of cell culture logistics. Various options are open for improving efficiency for cell culture or improved utilization of cellular reagents; for example, the application of microfluidic methods in order to reduce cell usage,¹⁷ or the bulk preparation of cell stocks with freezing of individual aliquots to be used as required.¹⁸ Microbead-based cell culture methods, where cells are cultured on beads maintained in proprietary tubes (LeviTube, Global Cell Solutions) represent an alternative to classical culture techniques. The microbeads are between 70 and 150 μm in diameter, which corresponds to a total surface area of 170 cm^2/mL of bead slurry (50% v/v).¹⁹ A single 50 mL consumable container could therefore replace multiple conventional T-175 flask containers, with the practical advantage of a smaller instrument footprint within the laboratory and potentially lower consumable costs. In our study, for a subset of the cell lines, we compared the performance of cells cultured and assayed on microbeads versus those cultured using a conventional T-flask.

MATERIALS AND METHODS

Isolation of Fungal Strains

Fungal strains were isolated from the North Sea (German Wadden Sea), from Mediterranean sponges, from Chilean algae, and from Indonesian corals. All strains were subsequently purified and stored at -80°C . Identification of fungal strains was based on morphological criteria, as well as genetic analysis of the internal transcribed spacer (ITS) region. DNA extraction was performed using the Pre-cellys 24 system (Bertin Technologies). Fungal-specific polymerase chain reaction (PCR) by amplifying the ITS1-5.8S rRNA-ITS2 fragment was carried out using puReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare) with the ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers.²⁰ PCR was conducted as follows: initial denaturation (2 min at 94°C), 30 cycles of primer denaturation (40 s at 94°C), annealing (40 s at 55°C), and elongation (1 min at 72°C) followed by a final elongation step (10 min at 72°C). PCR products were sequenced using the ITS1 primer. Sequence data were edited with ChromasPro v1.15 (Technelysium Pty Ltd.). Closest relatives were identified by sequence comparison with the NCBI Genbank database using the Basic Local Alignment Search

Tool (BLAST). Sequences were aligned using the ClustalX v2.0 software, and the alignment was refined manually using BioEdit v7.0.9.0. For alignment construction, ITS1-5.8S-ITS2 gene fragments from closest-cultured relatives according to BLAST as well as type strains were used whenever possible. Phylogenetic calculations were performed with all closest relatives according to BLAST results.

The following media were used for fungal culturing: WSP 30 (1.0% glucose $\cdot\text{H}_2\text{O}$, 0.5% peptone from soy meal, 0.3% malt extract, 0.3% yeast extract, 3.0% sodium chloride), Czapek dox (3% sucrose, 0.3% sodium nitrate, 0.1% di-potassium hydrogen phosphate, 0.05% magnesium sulfate, 0.05% potassium chloride, 0.001% ferric sulfate, 0.3% sodium chloride), GYM4 (0.4% glucose, 0.4% malt extract, 0.4% yeast), WM (1.0% glucose $\cdot\text{H}_2\text{O}$, 0.5% bacto peptone, 0.3% malt extract, 0.3% yeast extract, 3.0% tropic marine). TM indicates use of tropic marine sea salts instead of sodium chloride.

The cultures were inoculated from 14 d old precultures on agar plates and incubated using standing or shaking conditions at 28°C for a minimum of 14 d. Extracts for anticancer screening and metabolite composition analysis were prepared by extracting the whole cultures grown at 50–150 mL scale with an equivalent volume of ethyl acetate, concentrating to dryness and redissolving in an assay-compatible solvent (dimethyl sulfoxide [DMSO]) at a 100-fold concentration factor (*i.e.*, 0.5–1.5 mL).

For purification purposes, 10 L of medium were inoculated and cultured using the same conditions as for the screening. The culture filtrate was mixed with the same volume of ethyl acetate, and the organic solvent was separated and concentrated to dryness under reduced pressure.

Analyses of Extracts and Purification of Compounds

The extracts were dissolved in DMSO. An aliquot of 15 μL of the crude extract was analyzed by high-performance liquid chromatography-diode array detector coupled with electrospray mass spectrometry (HPLC-DAD/MS) for initial metabolic profiling. The crude extracts of 10 L cultures were subjected to preparative HPLC-UV (Phenomenex Gemini-NX C18 110A, 100 mm $\times$ 50.00 mm; eluents: H_2O and acetonitrile (ACN); gradient: 0 min 15% ACN, 26 min 86% ACN, 27 min 100% ACN; flow: 100 mL/min).

Dereplication and Structural Elucidation

Retention time, UV spectra, MS data, nuclear magnetic resonance (NMR) data, and activity of fractions were analyzed for common compounds using commercial and in-house databases.^{21,22}

Marine Compound Library

Following structural elucidation, the newly isolated compounds from this study were added to the well-established Kiel library of MNPs (KiWiZ collection). This resource includes a variety of known natural compounds of marine origin at a minimum purity of 80% and quantity of 10 mg. A group of 241 compounds was chosen from this KiWiZ library for inclusion in the part of the study where T-flask and microbead-based culture methods were compared.

Cell Culture for NCI Panel Profiling Using Classical T-Flasks

The NCI60 panel of cancer cell lines was maintained in RPMI-1640 medium containing 2 mM glutamine, 100 U/mL penicillin G, 100 µg/mL streptomycin, and 10% fetal calf serum (FCS). At about 80% confluence in T-flasks, cells were washed, trypsinized, and resuspended in RPMI-1640 medium before seeding into 384-well plates. Unless indicated otherwise (*e.g.*, microbead comparison study), all data reported here used conventional T-flask methods for cell production.

Cell Culture for NCI Panel Profiling Using Microbeads

The purpose of this part of the study was to compare results in T-flasks and microbead cell methods in order to identify an improved logistical setup for future routine screening activities. A549-ATCC, SF-539, and 786-0 cells were grown on Cytodex 1 (Sigma-Aldrich Co. LLC) beads, whereas the M14 cell line was cultured on Cytodex 3 (Sigma-Aldrich Co. LLC) beads in the BioLevigator system (Hamilton Bonaduz, serial no. 1006). Initial cell numbers were 2×10^6 M14 cells, 1×10^6 A549-ATCC cells, 3×10^6 SF-539 cells, and 7×10^6 cells in the case of 786-0. Inoculation was performed using agitation periods of 2 min without rotation pauses, 1 s rotation period, 60 or 50 (in the case of M14) rpm rotation speed, agitation pauses of 20 (for A549-ATCC) or 30 min, and 12 or 24 min (in the case of M14 and SF-539) agitation. For cultivation, all cells were incubated without rotation pauses, a rotation period of 3 s and a rotation speed of 60 rpm, except for A549-ATCC where a rotation speed of 70 rpm was used. The 786-0 cell line has to be maintained for less than 72 h due to potential clumping and detachment of cells, which can occur after prolonged cultivation times. For splitting, cells had to be detached from the microbeads, which proved challenging in the case of SF-539 cells. The beads with the other three cell lines were harvested, resuspended in 1% dextranase (1 vol; Sigma-Aldrich Co. LLC), and incubated for 10 min at 37°C in a 5% CO₂ atmosphere. Trypsin-EDTA solution (0.5 vol; GE Healthcare Bio-Sciences Corp.) was added, and the incubation continued for an additional 10 min at 37°C with 5% CO₂. Microbead cell culture methods were applied only to analysis of the 241 KiWiZ library compounds (not for the newly isolated compounds). The protocol is described in *Supplementary Table S2*.

Screening of MNPs Against the NCI Panel

Crude extracts obtained were profiled at three concentrations against 786-0, MCF-7, and M14 cell lines using the Neutral Red assay protocol described in *Supplementary Table S3* to identify those containing potentially useful anticancer compounds.²³ The 100-fold concentrated marine fungal fermentation extracts dissolved in DMSO were tested for antitumor activity at three different dilutions—(1) 1:200, (2) 1:1,000, and (3) 1:5,000—to facilitate ranking of activities detected based on potency. Active extracts were then subjected to an antitumor activity-guided fractionation approach using reversed phase HPLC, resulting, after two or three purification steps, in the isolation of single active compounds. These single compounds were then characterized in a wider range of cell lines from the NCI60 panel. Detailed screening on isolated compounds was conducted on a fully

automated cell::explorer system (Perkin Elmer), which included an Envision multimode reader (Perkin Elmer) and an Echo550 acoustic dispensing system (Labcyte).²⁴ Cells from T-flask culture methods were seeded (20 µL vol) at 500–2,500 cells per well (depending on cell line requirements) in 384-well microplates (CellStar white 384, polypropylene, tissue culture treated) and incubated at 37°C in the presence of 5% carbon dioxide (CO₂). At 24 h post seeding, the growth of baseline control plates was assessed using the CellTiter-Glo (CTG) protocol (Promega, Inc.). To each well, 20 µL of CTG detection mix was added, and plates were read on the Envision after 10 min. Assay plates were dosed with compound (11 concentrations in triplicate) using the Echo550 to transfer 100 nL of compound from source plates containing dilution series in 100% DMSO. The top compound concentration was 50 µg/mL (assay final). The DMSO concentration in the assay plates was uniform at 0.5% (final). Assay plates were then incubated for an additional 48 h before addition of the CTG detection reagents and reading. The relative growth at 72 h (vs. 24 h baseline) was used to calculate cell viability and proliferation parameters (GI₅₀, LC₅₀, and TGI), as described previously.²⁵ Data analysis used the ActivityBase software (IDBS Ltd.). For hit compounds, a counter assay was used to determine the influence of compounds on CTG performance. At 24 h post seeding, CTG detection reagents were added to plates followed by immediate addition of compound, and then read after 10 min. Data for counter assay studies were normalized relative to appropriate DMSO controls to give an “Apparent Activity” reading.

For the microbead comparison study, cell viability in the presence of the 241 compounds was assessed in an “on bead” assay format. The equivalent seeding densities were 2,000 cells per well for M14 cell line and 1,500 cells per well for A549-ATCC cells. In all other respects, the CTG viability assay protocol was identical to that employed using cells cultured in T-flasks. The separate group of 241 compounds was used for the microbead comparison study and was screened at 50 µM in singlicates, followed by dose–response studies on selected hits.

Label-Free Cytotoxicity Measurement Using Cell Metric

Label-free proliferation and viability assays were performed on the A549-ATCC cell line only (*Supplementary Table S4*) using the Cell Metric imaging system (Solentim). The instrument uses whole well brightfield imaging and automated image analysis. The properties of standard inhibitors (paclitaxel and cisplatin) were assessed. Experiments used clear bottom, black, 384-well cell carrier plates (Perkin-Elmer). Plates were measured (at 25°C) on days 1 and 3. The short read times (<3 min per plate) meant that extensive environmental control (humidity, CO₂, etc.) was not required, and readings could take place on a benchtop setting rather than in a cell incubator. The automated image analysis methods “Confluence” and “ViaConfluence” provided by the instrument manufacturer were applied. The morphological-based assessment operates on a cell-by-cell basis. Dead cells are differentiated from the live cell population by the presence of characteristic condensed nuclei. The output values were total cell number and the proportion of live and dead cells. Parallel CTG experiments were performed as described above.

Phospho Flow Analyses

Jurkat TAG cells were cultured under standard cell culture conditions in RPMI-1640, GlutaMAX, supplemented with 10% fetal bovine serum (FBS), MEM nonessential amino acids solution, sodium pyruvate (1 mM), and penicillin/streptomycin (Gibco, Life Technologies) in a 5% CO₂ atmosphere at 37°C. For the phospho flow analysis of the effects of marine fungal compounds on cellular signaling events, 250 µL cell suspension (5×10⁷ cells/mL) were pre-incubated with either DMSO or different concentrations of compounds (0.1 mM, 0.01 mM, and 0.001 mM) at 37°C for 1 h, followed by stimulation of the T-cell receptor (TCR) with 1.25 µg/mL OKT3 antibody (ATCC CRL-8001). At the indicated time points (0, 1, 5, and 15 min), 50 µL of cells was directly fixed in fix buffer I (BD Biosciences) and washed in phosphate-buffered saline (PBS). Afterwards, the cells were labeled with unique concentration combinations of the three fluorescent molecules Pacific Blue, Pacific Orange, and Alexa Fluor 488 following a 3D matrix setup (FCB), to enable merging of 48 different cell samples (3 different compounds×4 concentrations×4 time points) into one mixture.²⁶ In the FCB matrix, the different compounds were encoded by staining with distinct concentrations of amine-reactive Pacific Orange succinimidyl ester (500 ng/mL, 100 ng/mL, and 10 ng/mL). The time course was encoded using increasing concentrations of Pacific Blue succinimidyl ester (0 min 0.69 ng/mL, 1 min 6.25 ng/mL, 5 min 25 ng/mL, 15 min 100 ng/mL), and for the increasing compound concentrations, Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester was utilized (0 mM/DMSO only 0.69 ng/mL, 0.001 mM 6.25 ng/mL, 0.01 mM 25 ng/mL, 0.1 mM 100 ng/mL; Molecular Probes, Life Technologies). After FCB staining, the cells were washed in washing solution (PBS supplied with 1% FBS and 0.09% sodium azide), combined, permeabilized in perm buffer III (BD Biosciences), and stored in perm buffer III at –80°C until analysis. Then, the combined samples were thawed on ice, washed and stained with Alexa Fluor 647-coupled antibodies against CD3ζ (pY142), IgGκ, LAT (pY171), Lck (pY505), MEK1 (pS298), SLP76 (pY128), Stat1 (pY701), Stat3 (pY705), Stat5 (pY694), ZAP70/Syk (pY319/Y352) (BD Biosciences), Akt (pS473), Erk1/2 (pT202/Y204), Histone H3 (pS10), NF-κB (pS536), p38 (pT180/Y182), S6 ribosomal protein (pS235/S236), SAPK/JNK (pT183/185), phospho-tyrosine (pY100) (Cell Signaling Technology) and unconjugated antibodies against β-catenin (pS45), GSK3β (pS9), HSP27 (pS82), phospho-Akt substrate (RXXS/T), phospho-PKA substrate (RRXS/T), PLCγ-1 (pT783), (Cell Signaling Technology), c-Jun (pT91/T93) (Sigma), and Vav (pY174) (Santa Cruz Biotechnology). Samples stained with an unconjugated primary antibody were subsequently stained with Alexa Fluor 647 goat anti-rabbit IgG (H+L) or Alexa Fluor 647 goat anti-mouse IgG1 (γ1) (Molecular Probes, Life Technologies) secondary antibodies. After antibody staining, the samples were run on an LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star). The assay protocol is described in *Supplementary Table S5*.

Proximal T-cell signaling was covered by phospho-specific antibodies, and the three classical MAP kinase pathways were monitored via PLCγ-1 (pT783), MEK1 (pS298) and Erk1/2 (pT202/Y204), SAPK/JNK (pT183/185), and c-Jun (pT91/T93) as well as p38 (pT180/Y182)

and HSP27 (pS82). Stat signaling was assessed using Stat1 (pY701), Stat3 (pY705), and Stat5 (pY694). In addition, the panel contained NF-κB (pS536), an antibody for PKA activity (phospho-PKA substrate [RRXS/T]), an antibody detecting phosphorylated tyrosines in general (pY100), as well as a marker correlating with mitotic chromatin condensation, Histone H3 (pS10).²⁷ For all markers, fold changes of median fluorescence intensities (MFI) upon TCR stimulation were calculated compared to the 0 min sample of the respective compound concentration, whereas changes in basal phosphorylation levels were calculated comparing the 0 min samples of the different compound concentrations to the 0 min sample that was pre-incubated with DMSO only.

RESULTS

Strain Culture and Structural Elucidation

Fungal strains were isolated from Mediterranean sponges, Chilean macroalgae, Indonesian corals, and the German Wadden Sea and cultivated using WSP30, GYM4, WSP30TM, and Czapek Dox media employing static and shaken fermentation. From all of these combinations, fermentation extracts for screening were prepared as described in *Materials and Methods* and tested against the preliminary panel (for development and description, see *Supplementary Data*), which consists of a collection of four sensitive cell lines of the NCI60 panel. Screening of 1210 extracts against the preliminary panel detected 173 extract hits (defined as exhibiting cytotoxic or anti-proliferative activity at ≥1,000-fold dilution in the assay against ≥1 tumor cell line) from 110 different fungal strains. The activity value considered as hit was set deliberately weak, as it was not possible to take the relative amount of the active MNP within the fraction into account. This allowed detection of minor active compounds too. Some fungal strains produced activity in more than one fermentation medium or condition. Genetic analysis of the internal transcribed spacer (ITS) region led to the identification of the marine fungi. The identity of the 42 fungi with active extracts in the preliminary panel is given in *Table 1*.

Phenotypic Screening Strategy

The approach presented here to identify anticancer MNPs is based on a cellular phenotype instead of a molecular reaction and is therefore truly phenotypic. The workflow starts with the cultivation and activity-guided purification of marine fungi and their bioactive MNPs (*Fig. 1A*) using the preliminary panel of cancer cell lines (*Supplementary Data*). Two of the four cell lines—M14 and MCF-7—were used regularly in the Neutral Red assay employed in this screening process, and HL-60 was deprioritized in favor of 786-0 cell lines. The corresponding frequency distribution (in number of extracts) and the overall hit rate are shown in *Figure 1B and C*, respectively. As expected for a natural compound screening, the number of very active extracts represents only a small fraction. However, the hit rate of 14.3% in total is quite remarkable. Thirty-seven different fungi were selected for methodological setup and initial cultivation conditions leading to assay-guided purification. Inclusion of newly isolated fungi after testing their extracts into this

Table 1. Preselected Strains for Methodological Setup and Initial Cultivation Conditions

Sample	Identity (based on ITS sequences, next rel. acc. to BLAST)	Identity (%)	Source	Medium	Mode
KF082	<i>Fusarium oxysporum</i>	99	Algae	WSP30	Stand
KF120	<i>Pleosporales</i> sp. OY1507	92	Algae	WSP30	Shaken
KF120	<i>Pleosporales</i> sp. OY1507	92	Algae	WSP30	Stand
KF133	<i>Penicillium</i> sp.	99	Sponge	WSP30	Stand
KF482	<i>Penicillium</i> sp.	99	German Wadden Sea	Czapek Dox	Stand
KF482	<i>Penicillium</i> sp.	99	German Wadden Sea	Czapek Dox	Shaken
KF604	<i>Penicillium</i> sp.	98	German Wadden Sea	WSP30TM	Stand
KF620	<i>Penicillium viridicatum</i>	99	German Wadden Sea	WSP30TM	Stand
KF643	<i>Penicillium expansum</i>	100	German Wadden Sea	WSP30TM	Stand
KF666	<i>Botryotinia fuckeliana</i>	100	German Wadden Sea	WSP30TM	Stand
KF694	<i>Fusarium equiseti</i>	100	German Wadden Sea	WSP30	Stand
KF767	<i>Helicodendron paradoxum</i>	99	German Wadden Sea	WSP30TM	Stand
KF794	<i>Arthrinium sacchari</i>	98	German Wadden Sea	WSP30	Stand
KF823	<i>Fusarium tricinctum</i>	99	German Wadden Sea	WSP30	Stand
KF828	unidentified	Less than 80	German Wadden Sea	WSP30	Stand
KF839	<i>Penicillium canescens</i>	100	German Wadden Sea	WSP30	Stand
KF839	<i>Penicillium canescens</i>	100	German Wadden Sea	WSP30	Shaken
KF870	<i>Penicillium canescens</i>	99	German Wadden Sea	WSP30	Stand
LF037	<i>Penicillium brevicompactum</i>	100	Sponge	WSP30	Stand
LF054	<i>Verticillium luteoalbum</i>	100	Sponge	WSP30	Stand
LF066	<i>Penicillium chrysogenum</i>	100	Sponge	WM	Stand
LF098	<i>Penicillium chrysogenum</i>	100	Sponge	WSP30	Stand
LF245	<i>Fusarium equiseti</i>	100	Sponge	GYM4	Stand
LF363	<i>Penicillium rugulosum</i>	99	Sponge	WSP30	Stand
LF363	<i>Penicillium rugulosum</i>	99	Sponge	WSP30	Stand
LF381	<i>Penicillium chrysogenum</i>	100	Coral	WSP30	Stand
LF432	<i>Penicillium citreonigrum</i>	100	Sponge	WSP30	Stand
LF547	<i>Aspergillus flavipes</i>	99	Sponge	WSP30	Stand
LF584	<i>Aspergillus ustus</i>	99	Sponge	WSP30	Stand
LF590	<i>Penicillium citreonigrum</i>	100	Sponge	WSP30	Stand
LF596	<i>Penicillium canescens</i>	100	Sponge	WSP30	Stand

(continued)

Table 1. (Continued)

Sample	Identity (based on ITS sequences, next rel. acc. to BLAST)	Identity (%)	Source	Medium	Mode
LF607	Uncultured ascomycete sp.	98	Sponge	WSP30TM	Shaken
LF615	<i>Verticillium</i> sp.	98	Sponge	WSP30	Stand
LF627	<i>Aspergillus versicolor</i>	100	Sponge	WSP30TM	Stand
LF634	<i>Penicillium expansum</i>	100	Sponge	WSP30	Stand
LF652	<i>Aspergillus versicolor</i>	100	Coral	WSP30	Shaken
LF852	<i>Penicillium expansum</i>	99	Algae	Czapek Dox	Shaken

Identification of fungal strains based on morphological criteria as well as on genetic analysis of the internal transcribed spacer (ITS) region. Closest relatives to fungal strains according to BLAST search are presented.

Fig. 1. Experimental setup and screening strategy to identify anticancer compounds derived from marine fungi. **(A)** Fungi were isolated from various marine habitats extracted by means of ethyl acetate and subsequently analyzed by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-MS). Fractions were tested for activity in up to four sensitive cell lines and purified to single chemical entities. Pure compounds from the Kiel Library of Natural Compounds and from the marine extracts were analyzed in the screening toolbox to assess activity and specificity. **(B)** Frequency distribution of relative cellular growth of 1,210 marine extracts against M14 cell line (dark gray) and MCF-7 cell line (light gray). **(C)** Summary of results of extract screening against the preliminary panel. A hit is defined as an extract exhibiting cytotoxic or antiproliferative activity at $\geq 1,000$ -fold dilution in the assay against ≥ 1 tumor cell line. Screening was done in $n = 3$ replicates, and mean values were used for hit identification. Color images available online at www.liebertpub.com/adt

process is still an ongoing process, with 173 compounds purified and identified to date submitted to the phenotypic screening toolbox. Together with the 241 compounds of the KiWiZ library, a total of 414 compounds were subjected to the phenotypic screening toolbox. By using HTS-compatible cytotoxicity profiling with image-based and bioinformatics analysis and detailed FACS analysis (Fig. 1A), the full spectrum from phenotypic activity profiling to target identification could be performed as presented.

Profiling Using Selected Cell Lines From the NCI Panel

The assay development process focused on establishing the DMSO tolerance, optimal cell seeding density, pharmacology for standard compounds, and minimizing interexperiment variability. Representative results from the assay development process are shown in Figure 2. The 0.5% DMSO level used in screening was well tolerated for the NCI cell lines (Fig. 2A). The response of the CTG assay to cell number, in the presence and absence of 0.5% DMSO, was linear at low seeding densities (Fig. 2B and inset). The assay response of standard compounds (cisplatin, 6-mercaptopurine, paclitaxel, and staurosporine; Fig. 2C) was broadly consistent with data held in the Development Therapeutics Program databases. With the M14 cell

line, values for ($\log_{10}$ GI₅₀, LC₅₀, and TGI) were: cisplatin (−4.49, −4.21, and −3.97); 6-mercaptopurine (−8.33, −8.13, and −7.82); paclitaxel (−8.37, −7.87, and −7.22); and staurosporine (−7.51, −6.89, and −6.28). As illustrated in Figure 2D, cisplatin, however, appears to influence the CTG detection reagents >100 μ M. The effect of cisplatin in the counter assay is only apparent, however, at compound concentrations well in excess of those where cell growth and viability are affected. Other standard compounds were clear in the counter assay.

All assays were quality controlled with respect to performance of control compounds and a cutoff Z' value of 0.6 was set as a threshold for quality control during production screening, although typically Z' values exceeded 0.75. A counter assay to monitor effects on the CTG detection reagents was performed on all active compounds.

Studies on extracts used assay-guided fractionation and, based on their metabolite profiles determined by HPLC-DAD/MS, 37 metabolites were then isolated from cultures of a subset of these fungi (Table 2). The isolated MNP compounds inhibited both cell growth and viability over the concentration range investigated. Structural elucidation was undertaken in parallel with detailed profiling

of purified compounds. Structures of representative active compounds are given in Figure 3. Representative dose response series for two active compounds—secalonic acid D and bisdethiobis(methylthio)gliotoxin—are shown in Figure 4B and C, respectively. The distribution of compound activities (GI₅₀, TGI, and LC₅₀) against 786-O, MCF-7, M14, and HL-60 cell lines (cultured using T-flask) for the 37 compounds are shown in Figure 4A (see Supplementary Table S8 for details of curve fit results). All 37 test compounds were clear in the counter assay (data not shown).

Microbead Culture and “On-Bead” Assays

The BioLevigator microbead cell culture with the “on-bead” CTG assay gave responses for the standard compounds, which were similar to that seen with cells (M14) cultured using conventional methods (Table 3). With cell line A549-ATCC (Fig. 5A), the values for ($\log_{10}$ GI₅₀, LC₅₀, and TGI) were cisplatin (−4.23, −3.83, and −3.44), 6-mercaptopurine (−5.91, −4.86, and −1.05), paclitaxel (−7.49, −5.86,

Fig. 2. Assay development using the NCI60 panel of cancer cell lines. (A) Dimethyl sulfoxide (DMSO) tolerance study for cell line 786-o. (B) Titration of the cell number using 786-o without DMSO (black circles) and 0.5% DMSO (gray cross); inset shows the linear response range. (C) Response of M14 cell line to the four standard compounds cisplatin (continuous black line with crosses), 6-mercaptopurine (continuous gray line with triangles), staurosporine (dashed black line with circles), and paclitaxel (dashed gray line with diamonds). (D) Effects of cisplatin on percent cell growth of M14 cell line (continuous black line with crosses) and apparent activity in counter assay (continuous gray line with crosses). The data presented are means $\pm$ standard error of $n=5$ values.

Table 2. Compounds Isolated From Preselected Strains After Cultivation as Shown in Table 1

Sample	Identity (based on ITS sequences, next rel. acc. to BLAST)	Compound
KF082	<i>Fusarium oxysporum</i>	Trichosetin
KF120	<i>Pleosporales</i> sp. OY1507	Herbarin
KF120	<i>Pleosporales</i> sp. OY1507	Scorpinone
KF133	<i>Penicillium</i> sp.	Cladosporin
KF482	<i>Penicillium</i> sp.	Macrophorin D
KF482	<i>Penicillium</i> sp.	O-Methylisogrifolin
KF604	<i>Penicillium</i> sp.	Cyclo(Tryptophyl-Tryptophyl-)
KF620	<i>Penicillium viridicatum</i>	Eutypoid B
KF643	<i>Penicillium expansum</i>	Communesin B
KF666	<i>Botryotinia fuckeliana</i>	Alternariol
KF694	<i>Fusarium equiseti</i>	Fusarochromanone
KF767	<i>Helicodendron paradoxum</i>	Fusaquinone C
KF794	<i>Arthrimum sacchari</i>	Hydroxyxanthinone
KF823	<i>Fusarium tricinctum</i>	Enniatin
KF828	unidentified	Terpestacin
KF839	<i>Penicillium canescens</i>	Demethoxygriseofulvin
KF839	<i>Penicillium canescens</i>	Penitrem A
KF870	<i>Penicillium canescens</i>	Penigequinolone A or B
LF037	<i>Penicillium brevicompactum</i>	Mycophenolic acid
LF054	<i>Verticillium luteo-album</i>	Tenuazonic acid
LF066	<i>Penicillium chrysogenum</i>	Secalonic acid D
LF098	<i>Penicillium chrysogenum</i>	Xanthocillin X
LF245	<i>Fusarium equiseti</i>	T-988B
LF363	<i>Penicillium rugulosum</i>	Skyrin
LF363	<i>Penicillium rugulosum</i>	Rugulosin
LF381	<i>Penicillium chrysogenum</i>	Member of the bisorbicillinol family
LF432	<i>Penicillium citreonigrum</i>	Bisdethiobis(methylthio)gliotoxin
LF547	<i>Aspergillus flavipes</i>	(2'E,4'E,6'E)-6-(1'-carboxyocta-2', 4',6'-trien)-9-hydroxydrim-7-ene-11,12-olide
LF584	<i>Aspergillus ustus</i>	WIN-66306

(continued)

Table 2. (Continued)

Sample	Identity (based on ITS sequences, next rel. acc. to BLAST)	Compound
LF590	<i>Penicillium citreonigrum</i>	Citreoviridin A or C
LF596	<i>Penicillium canescens</i>	Tryptotoquivaline
LF607	Uncultured ascomycete sp.	Isochromophilone IV or VIII
LF615	<i>Verticillium</i> sp.	Glriosein
LF627	<i>Aspergillus versicolor</i>	Notoamide D
LF634	<i>Penicillium expansum</i>	Butyrolactone I
LF652	<i>Aspergillus versicolor</i>	5-Methoxysterigmatocystin or aspertoxin
LF852	<i>Penicillium expansum</i>	Hydroxyterphenyllin

and no-fit), and staurosporine (−6.74, −5.72, and −5.16). The exception was paclitaxel, which for A549-ATCC cells failed to show the expected cytotoxic effects (negative cell growth) and exhibited an enlarged appearance at elevated concentrations. For the 241 KiWiZ library compounds screened against the A549-ATCC cell line, the correlation between 2D and microbeads was >0.95, demonstrating a high degree of comparability of the two protocols (Fig. 5B).

Label-Free Viability Assay

Representative time series images are shown in Figure 6A for the A549-ATCC cell line. Cell metric images at 24 h (precompound addition) and 72 h (48 h post cisplatin or paclitaxel addition) were assessed using the “Confluence” algorithm (Solentim). It can be seen with cisplatin that there is a good correlation between CTG and image-based label-free assays (Fig. 6C). However, in the case of paclitaxel, the label-free method identifies a cytotoxic effect (negative relative growth values) at higher concentrations, which is absent in the CTG results (Fig. 6D).

Application of Phospho Flow Cytometry

To evaluate the effects of compounds on cellular signaling cascades, we applied phospho flow cytometry in combination with FCB using the Jurkat T cell leukemia cell line as a model (Table 5).²⁸ Samples from the various conditions were labeled with different staining intensities of the three different fluorescent dyes Pacific Blue, Pacific Orange, and Alexa Fluor 488 (FCB), which enabled combination of 48 different samples into one pooled FCB-labeled mixture of samples (Fig. 7A–C) that could be deconvoluted to individual samples again during the subsequent analysis after flow cytometry (Fig. 7D–I). The FCB-labeled combination of samples was permeabilized, intracellularly stained with a panel of phospho-specific antibodies, and analyzed by flow cytometry. The antibody

Fig. 3. Secondary metabolites isolated from marine fungal strains and tested in various assays reported here. (A) Trichosetin, (B) rugulosin, (C) secalonic acid D, (D) mycophenolic acid, (E) terpestacin, (F) calcaride A, (G) isochromophilone IV or VIII (epimers), (H) herbarin, (I) penitrem A, (J) (2'E, 4'E, 6'E)-6-(1'-carboxyocta-2',4',6'-trien)-9-hydroxydrim-7-ene-11,12-olide, and (K) fusarochromanone. As absolute configuration was not determined for the majority of the compounds, no stereochemistry is shown in the figure. Further details of selected compound activities can be found in the *Supplementary Data*.

panel was designed to cover proximal TCR signaling, as well as further non-T-cell-specific downstream pathways that are shared by a variety of different cell types and known to be involved in cancer development and progression. That covered readouts for MAP kinase, Akt, mTOR, NF- κ B, and Stat signaling pathways that also process signals from growth factors such as EGF (RTKs), growth hormones, GPCRs, and cytokine receptors (Fig. 7J).^{29–31}

Calcaride A (Fig. 3F) analyzed by this detailed signal pathway analysis inhibited the prosurvival and proliferation signaling usually induced by TCR stimulation, as could be clearly observed at the highest compound concentration (0.1 mM).³² The activation of the MAP kinases Erk1/2 in the mitogenic Ras-Raf-MEK-Erk signaling pathway displayed delayed phosphorylation kinetics and a lower phosphorylation maximum of T202/Y204 (Fig. 7L). After 1 min, phosphorylation in untreated cells was increased approximately sixfold, whereas the compound-treated sample only showed a threefold change. The maximum, reached after 5 min, was decreased from an average of sevenfold change to sixfold. In addition, phosphorylation of S6 ribosomal protein at S235/S236, a readout for the Akt-mTOR survival signaling pathway, was decreased from a 6 $\times$ change to a 1 $\times$ change in the compound treated sample after 15 min stimulation (Fig. 7M).

DISCUSSION

Many oncogenic events are multifactorial in origin, which presents considerable challenges using a target-centric screening method when seeking to identify starting points for new therapeutics. The use of phenotypic methods based on an efficacy-related endpoint offers an alternative approach, especially when used in conjunction with the NCI60 cell line panel leading to significant advantages in the search for anticancer agents.

A phenotypic assay process was established and validated in order to identify compounds with anticancer properties from a variety of extracts and pure compounds produced by marine fungi.

The cell growth and viability screening was adapted to increased throughput and a higher degree of automation whilst maintaining the sensitivity. The assay was performed in a 384-well format, with 20 μ L total volume making it compound and reagent efficient. The sen-

sitivity of the CTG assay, even at low cell seeding densities (<500 cells per well), offers the advantage of reducing the cell culture load for profiling efforts compared to alternative protocols where typically 10 times the number of cells used here are required. Although there are substantial operational benefits to the use of CTG, it has been noted recently that ATP detection assays may underestimate the potency of compounds with certain modes of action due to compounds inducing changes in cell size (leading to greater mitochondrial mass) or modulating underlying mitochondrial activity.³³ Our study on A549-ATCC cells (Figs. 5A and 6D) and the work of others³³ have shown that paclitaxel cytotoxicity is underestimated by ATP quantification methods, likely in part due to these mechanisms. Here, we have shown that the use of label-free whole plate imaging to identify live and dead cell populations has the potential to detect compound activities that are not accessible to ATP-based methods (Fig. 6D). An advantage of label-free methods in the context of screening for anticancer agents is that, as a nondestructive readout, it will be possible to track the kinetics of compound effects on cell proliferation and viability. Establishing a better understanding of the time dependency of the onset of compound effects is of particular relevance when considering the pharmacokinetic and pharmacodynamic properties of the compounds during later *in-vivo* stages.

Fig. 4. The effect of isolated MNP compounds on cell growth. **(A)** Frequency distribution of all LC₅₀, TGI, and GI₅₀ values (μM) for 37 compounds against the same four cell lines LC₅₀ (black filled), TGI (gray filled), and GI₅₀ (white filled). **(B)** Normalized cell growth dose-response curves for secalonic acid D against four cell lines (786-o, MCF-7, M14, and HL-60). **(C)** Normalized cell growth dose-response curves for bisdethiobis(methylthio)gliotoxin against four cell lines (786-o, MCF-7, M14, and HL-60). See *Supplementary Table S1* for source data and details of curve fit results. The data presented are means ± standard error of $n=3$ values.

A good correlation was obtained between compound activities (241 from the KiWiZ library) measured against cell lines cultured under conventional 2D cell methods versus cells produced using microbead culture methods and assayed “on-bead” (Fig. 5 and Table 3). The 241 compounds in our test set exert their anticancer activities via a wide range of potential mechanisms. The correlation observed indicates that the targets associated with cancer phenotypes are independent of these two culture methods for the A549-ATCC cell line. This contrasts with multiple observations of “classical” 3D culture systems, involving scaffolds such as Matrigel, where cell morphology, differentiation, and cell-matrix interactions are modified, and consequently the cells are considered to be better able to represent the *in-vivo* state.³⁴ For example, malignant human breast epithelial cells in Matrigel cultures exhibit a normal morphology, which is mediated by cell-matrix interactions, leading to the inhibition of epidermal growth factor receptor (EGFR) and β1-integrin

signaling.³⁵ It is widely recognized that 3D models can offer a superior test model for anticancer agents. However, our data would suggest that the microbead system performance in terms of biological relevance is more closely aligned with that of 2D cell culture methods. In initial studies, once cell coverage exceeded 80% on the surface of the microbeads, the rate of cell growth was greatly reduced (data not shown), which made cell growth inhibition type assay readouts difficult to access due to the minimal window. For this reason, at time zero, beads were seeded at only 20%–30% surface cell coverage. Therefore, target mechanisms mediated by cell-cell interactions are likely not to be enhanced in the microbead culture format, which would suggest that it cannot be used as a substitute for 3D culture methods. However, a higher degree of automation efficiency is possible using the microbead culture method, as it allows straightforward optimization of cell number to growth media ratios and improved general management of the cell culture process, including more efficient and less disruptive cell handling. In particular, as we have shown that assays can be performed “on-bead” (Table 3 and Fig. 5A). Therefore, potentially harsh cell harvesting techniques using proteolytic agents such as trypsin are not required.

In this study, we have characterized different fungi of a variety of marine origins and structurally elucidated their active secondary metabolites. In common with reported studies on other microbes, the choice of the medium had a substantial influence on secondary metabolite production. For example, most compounds shown in Figure 3 were identified from fungi cultivated in WSP30 medium (e.g., trichosetin and rugulosin; Fig. 3A and 3B) or in WSP30 supplemented with marine sea salts (isochromophilone; Fig. 3E), while only secalonic acid D was identified from a fungus cultivated in WM medium (Fig. 3C). The majority of the more potent compounds reported here has been previously identified as having anticancer or general cytotoxic activities. For example, secalonic acid D has been

Table 3. Comparison Between T-Flask and Microbead Cell Culture Methods Using M14 Cells

	Log ₁₀		
	GI ₅₀	LC ₅₀	TGI
Cisplatin			
T-flask	4.49	4.21	3.97
Microbead	3.72	3.54	3.33
Paclitaxel			
T-flask	8.37	7.87	7.22
Microbead	8.08	7.77	7.14

Fig. 5. Comparison of microbeads and 2D cell culture. **(A)** Response of A549-ATCC cell line to the four standard compounds: cisplatin (continuous black line with crosses), 6-mercaptopurine (continuous gray line with triangles), staurosporine (dashed black line with circles), and paclitaxel (dashed gray line with diamonds). **(B)** Normalized cell growth, comparison of 2D and microbead cell culture with A549-ATCC cells using 241 MNP's from the KiWiZ library. The data presented are means $\pm$ standard error of $n=5$ values.

shown to cause cell cycle arrest of G(1) phase and downregulation of c-Myc through activation of GSK-3 β .³⁶ Reported IC₅₀ values against HL60 and K562 cells were 0.38 and 0.43 μ M, and are similar to TGI values obtained in this study. Similarly, bisdethiobis (methylthio)gliotoxin isolated from the marine-derived fungus *Penicillium* sp. has been reported to have potent cytotoxic activity and methyltransferase inhibiting properties.³⁷ Nevertheless, the ability of the platform to identify these previously reported compounds demonstrates the robustness of the whole process from harvesting of the producing organism through to the *in vitro* characterization of the activities of the active secondary metabolites. This MNP drug discovery platform has also been successful in characterizing a series of novel compounds such as the calcarides,³² represented by calcaride A in Fig. 3F, which acts in a biologically relevant manner as evidenced by phosphor flow experiments showing MAP kinases Erk1/2 activation in the cancer-relevant mitogenic Ras-Raf-MEK-Erk signaling pathway (Fig. 7). These and other novel compounds are currently under more detailed investigation, and for selected examples, their activity in *in vivo*

settings will be determined as part of future studies. Hence, the established phenotypic toolbox can be used for identification of active compounds within a bioassay-guided purification approach, as well as throughout characterization of pure compounds available, for example, in compound libraries. The advantage of this phenotypic toolbox is the higher probability of hit finding compared to single-target approaches.

Following the identification of active compounds using phenotypic readouts, there will be a chance in future studies to provide a preliminary assessment of the underlying targets. The NCI has established the online tool COMPARE to facilitate

this analysis.¹¹ A statistical comparison is made between the growth characteristic pattern produced by unknown compounds and a database containing the activity of known compounds. The wealth of

Fig. 6. Label-free image-based cell viability and proliferation assay assessment with A549-ATCC cell line. **(A)** Brightfield images of a DMSO (0.5%) treated cells at 3, 24, 48, and 72 h post seeding. **(B)** Confluence analysis of images in **(A)**. **(C)** Comparison of cell growth for cisplatin using CTG (gray diamond) or image-based (black circle) analyses. **(D)** Comparison of relative cell growth for paclitaxel using CTG (gray diamond) or image-based (black circle). The data presented are means $\pm$ standard error of $n=5$ values.

Fig. 7. Phospho flow cytometry in combination with fluorescent cell barcoding (FCB). (**A–E**): Schematic overview of a phospho flow experiment using one-dimensional FCB. Cells in suspension are stimulated (**A**), fixed, stained with different intensities of a fluorescent dye (**B**), and combined into one mixture of FCB-labeled samples (**C**). After permeabilization and antibody staining, the cells are analyzed by flow cytometry. The different barcoded cell populations can be deconvoluted based on their distinct staining intensities during the analysis (**D**) and then be analyzed separately (**E**). (**F–I**): Gating strategy for an experiment with a three-dimensional (3D) FCB of 48 samples. First, cells are selected based on forward (FSC) and sideways scattered light (SSC) (**F**), followed by doublet discrimination (**G**) and deconvolution of the 3D staining matrix (**H**, **I**). (**J**) Overview of the pathways analyzed. Signaling molecules detected via antibodies are highlighted in gray, S6rp: S6 ribosomal protein, β -Cat: β -catenin. (**K–M**): Effects of calcaride A on signaling. (**K**) Isotype control (IgGk), (**L**) Erk1/2 (pT202/Y204), and (**M**) S6 ribosomal protein (pS235/S236). The data presented are means $\pm$ standard error of $n=3$ values.

proteomics and transcriptomics data associated with the NCI60 panel also provides additional benefits in compound profiling decision making and prioritization.^{38,39}

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No competing financial interests exist.

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Abbreviations Used

2D = two-dimensional
 3D = three-dimensional
 ACN = acetonitrile
 ATP = adenosine triphosphate
 BLAST = Basic Local Alignment Search Tool

CTG = CellTiter-Glo
 DMSO = dimethylsulfoxide
 FACS = fluorescence activated cell sorting
 FCB = fluorescent cell barcoding
 FCS = fetal calf serum
 FSC = forward-scattered light
 GI₅₀ = half-maximal growth inhibition
 HPLC = high pressure liquid chromatography
 HTS = high throughput screening
 ITS = internal transcribed spacer
 KiWiZ = Kieler Wirkstoffzentrum
 LC₅₀ = half-maximal lethal concentration
 MFI = median fluorescence intensity
 MNP = marine natural product
 NCI = National Cancer Institute
 NIH = National Institutes of Health
 NMR = nuclear magnetic resonance
 PCR = polymerase chain reaction
 SSC = sideways-scattered light
 TCR = T-cell receptor
 TGI = total growth inhibition