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RO0504985 is an inhibitor of CMGC kinase proteins and has anti-human cytomegalovirus activity

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ABSTRACT

Public-private partnerships allow many previously unavailable compounds to be screened for antiviral activity. Here a screening method was used to identify an oxindole compound, RO0504985, from a Roche kinase inhibitor library that inhibited human cytomegalovirus (HCMV) protein production. RO0504985 was previously described as an inhibitor of cyclin-dependent kinase 2 (CDK2). However, using kinase selectivity assays it was found that RO0504985 was an inhibitor of several CMGC group kinase proteins, including CDK2. Using virus yield reduction assays it was observed that RO0504985 inhibited replication of different HCMV strains at low micromolar concentrations. Western blotting was used to investigate how RO0504985 inhibited HCMV replication. Treatment of HCMV infected cells with RO0504985 inhibited production of the immediate early viral IE2 proteins and the late viral protein pp28. Thus, RO0504985 inhibited HCMV replication by preventing production of specific HCMV proteins necessary for virus replication.

1. Introduction

Several factors, including bioavailability, drug resistance and toxicity, limit the use of currently available anti-human cytomegalovirus (HCMV) drugs (Biron, 2006; Coen and Schaffer, 2003; Mercorelli et al., 2008; Schleiss and McVoy, 2004; Villarreal, 2003). While several novel anti-HCMV drugs are now under development (Coen and Schaffer, 2003; Mercorelli et al., 2008), it remains necessary to expand our understanding of what compounds have anti-HCMV activity.

Screening of compound libraries is a useful methodology to survey compounds for potential anti-viral activity. However, the number of libraries currently available for screening is limited. Plus, there is often little diversity of chemical structures within those collections and the compounds within each library are often poorly characterised (Drewry et al., 2014; Knapp et al., 2013).

Public-private partnerships are one route to expand the number of compounds available for screening (Drewry et al., 2014; Knapp et al., 2013). Here, a screening methodology was used to identify potential anti-HCMV compounds within a Roche kinase inhibitor library. This collection is comprised of a diverse range of previously reported compounds that inhibit kinase proteins from several proteins kinase groups.

2. Materials and methods

2.1. Compounds

A Roche Kinase Inhibitor library (Table S1) was supplied to the Institute of Chemistry and Chemical Biology-Longwood (Harvard Medical School) by Hoffmann-La Roche Inc. All compounds were resuspended in dimethyl sulfoxide (DMSO).

2.2. Cells and viruses

Human foreskin fibroblast (HFF) cells (clone Hs29) were used. HCMV strains AD169 and Merlin RCMV1111 (Stanton et al., 2010)

Abbreviations: CDK, cyclin-dependent kinase; CK, casein kinase; CLK, cdc2-like kinase; CMGC, kinase group named using the initials of several members of that group; DYRK, dual specificity tyrosine-phosphorylation-regulated kinase; GSK, glycogen synthase kinase; HIPK, homeodomain-interacting protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MOK, MAPK/MAK/MRK overlapping kinase; MSSK, muscle-specific serine kinase; NLK, Nemo-like kinase; SAPK, stress-activated protein kinase.

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were gifts from Don Coen (Harvard Medical School) and Richard Stanton (Cardiff University), respectively.

2.3. Screening of compounds

See Supplementary Material.

2.4. Viral yield reduction

HFF cells were plated at a density of 5×10^4 cells per well. After 24 h incubation, cells were infected with HCMV at a multiplicity of infection (MOI) of 1. After virus adsorption for 1 h at 37 °C, cells were washed and incubated with 0.5 ml of media containing different dilution series of DMSO or RO0504985 that covered a range of concentrations from above 10 μM to below 0.01 μM. Plates were incubated for 96 h at 37 °C. Titers were determined by serial dilution of viral supernatant onto HFF monolayers which were covered in DMEM media containing 10% FBS and 0.6% methylcellulose. Cultures were incubated for 14 days, cells were stained with crystal violet and plaques were counted. The final concentration of DMSO in all samples was maintained at <1% (v/v).

2.5. MTT assays

HFF cells were seeded at a density of 1×10^4 cells per well. After overnight incubation to allow cell attachment, cells were treated for 96 h with a dilution series of DMSO or RO0504985 that covered a range of concentrations from 10 μM to below 0.1 μM. Cytotoxicity or inhibition of cell division was then determined with an MTT assay (GE Healthcare) according to the manufacturer's protocol, in which the ability of NAD(P)H-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan was assayed. The final concentration of DMSO in all samples was maintained at <1% (v/v).

2.6. Kinase selectivity analysis

RO0504985 was submitted to the Eurofins Pharma Discovery (Dundee) KinaseProfiler™ service to assay the ability of 1 μM of compound, or an equivalent volume of DMSO, to inhibit the panel of 43 CMGC kinase proteins shown in Fig. 2. Full details of each kinase protein inhibition assay can be obtained by contacting Eurofins Pharma Discovery. Briefly, recombinant protein kinases were tested for their ability to inhibit removal of a radiolabeled phosphate from γ-33P-ATP. Each *in vitro* kinase reaction was initiated by adding of 10 μM MgATP. After 40 min incubation at room temperature, reactions were stopped by adding 3% phosphoric acid. Ten μL of each reaction was spotted onto Filtermat and washed (three times for 5 min in 75 mM phosphoric acid and once in methanol) prior to drying and scintillation counting.

2.7. Western blotting

At time points indicated in the Figure cells were washed with PBS and resuspended in Laemmli buffer containing 5% β-mercaptoethanol. Proteins were separated on 8% or 10% polyacrylamide gels. Membranes were probed with antibodies recognizing IE1/2, pp28, pp65, UL44, UL84, (Virusys, 1:1000 dilution), IE2 proteins (clone 5A8.2, Millipore, 1:1000 dilution), UL85, UL86 (both kind gifts from Wade Gibson, Johns Hopkins University School of Medicine, 1:1000 dilution) and β-actin (SIGMA, 1:5000 dilution). All primary antibodies were detected using anti-mouse- or anti-rabbit-horseradish peroxidase (HRP) conjugated antibodies (Millipore and Cell Signaling Technology, respectively. Used at 1:10,000 and 1:2000 dilution, respectively.). Chemiluminescence solution (GE Healthcare) was used to detect secondary antibodies on film. Where shown, the presence of β-actin was used as a control to demonstrate similar amounts of cell lysate were assayed. Where indicated, relative band intensity (band intensity relative to β-actin signal in the same lane) was analysed using ImageJ software, obtained from the National Institutes of Health (USA).

3. Results and discussion

3.1. Kinase inhibitor screening

A Roche Kinase Inhibitor library of 235 compounds (Table S1) was screened at a concentration of 9.4 μM to identify compounds that inhibited production of the HCMV protein pp28 in cells infected with high passage strain AD169 (Wilkinson et al., 2015). Eighteen compounds (Table S2) produced a notable decrease in the number of cells in the assay (see Supplementary Material for criteria) and were judged to be cytotoxic to HCMV infected cells. Remaining data from the screen (217 compounds) was then converted to a z-score (the number of standard deviations from the mean of the data (Birmingham et al., 2009; Zhang et al., 1999)) to show an increase or decrease (positive or negative z-score, respectively) in the number of pp28 positive cells (Fig. 1A and Table S3).

There was no obvious overall relationship between compound structure or putative compound target and the positive or negative z-score assigned to each compound, except for 5 structurally related quinolinyl-methylene-thiazolinone compounds (RO4600445, RO4569139, RO4509200, RO4915610 and RO4554339) that had negative z-scores of −2.0 to −2.3 (Figs. 1A and B and Table S3). Using MTT assays it was found that these quinolinyl-methylene-thiazolinone compounds had 50% cellular

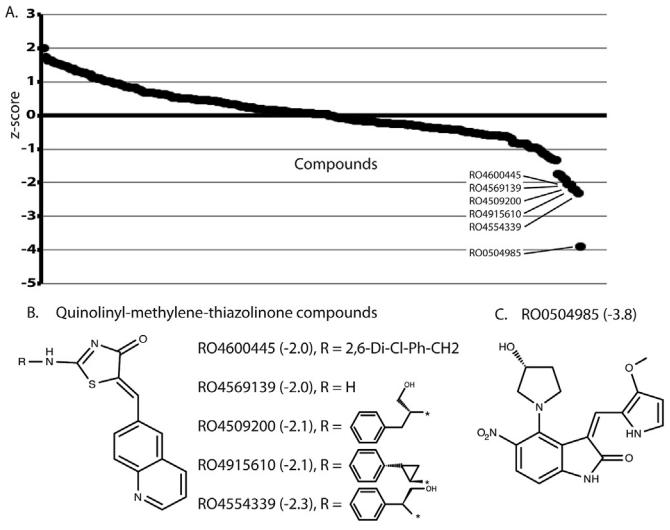

Fig. 1. Compounds assigned z-scores. (A) The ability of compounds within the Roche Kinase collection to inhibit HCMV strain AD169 protein production in HFF cells was assessed using the screen described in Materials & Methods. After exclusion of compounds judged to by cytotoxic, each compound was assigned a z-score (the number of standard deviations from the mean value of the screen) to describe the number of cells expressing viral antigen pp28 Thus, negative and positive z-scores represent fewer or greater numbers of cells expressing pp28, respectively. A plot of all z-scores is shown, where each data point represents a single compound. A list of each compound with its assigned z-scores is shown in Supplementary Table S3. The z-scores of several compounds discussed in the text are highlighted. (B and C) The structures and z-scores (stated in parentheses) of quinolinyl-methylene-thiazolinone compounds and RO0504985, respectively.