Supplementary Data

Inhibition of dynamin by dynole™ 34-2 induces cell death following cytokinesis failure in cancer cells

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Supplementary Materials and Methods

Drugs

The active dynamin inhibitors, dynoles™ (Ascent) were prepared as 30 mM stock solutions in DMSO and stored at -20°C. Prior to further dilution in the assay, the drugs were diluted in 50% (v/v) DMSO/20 mM Tris-HCl pH 7.4 or cell culture media and stored at -20°C. The final concentration of DMSO in the GTPase assay was 1%.

Protein Production

Native dynamin I was extracted and purified from the peripheral membrane fraction of whole sheep brain by affinity purification on GST-AmphII-SH3-Sepharose as described previously (1). The yield of dynamin I protein from 250 mg sheep brain is 8-10 mg. Rat full length dynamin II (His-6-tagged) DNA inserted into the pIEx-6 vector was generously provided by Sandra Schmid and Sylvia Neumann (The Scripps Research Institute). The cell culture and transfection procedures for dynamin II were carried out by Louis Lu and Tram

Phan from NCRIS-recombinant facility (CSIRO, Melbourne). The plasmid was used to transfect Sf21 insect cells using polyethylenimine (Polysciences) as the transfection reagent with a DNA/polyethylenimine ratio of 1:5 for 48 h. The cells were harvested and lysed following transfection, and overexpressed dynamin II was purified using GST-AmphII-SH3-sepharose as described above for dynamin I. The yield of dynamin II from 1 L of insect cell suspension culture (2 x 10^6 cells/ml) was typically ~10 mg protein with ~98% purity.

Malachite Green GTPase Assay

Malachite green dye was used for the sensitive colorimetric detection of orthophosphate (Pi), based on stimulation of purified dynamin by sonicated phosphatidylserine (PS) liposomes (1). For comparison of the IC_{50} values for dynI and II the assay components were modified to be performed under identical conditions of enzyme concentration, liposome and GTP concentrations. Purified dynamin I (49 nM) or dynamin II (49 nM) (diluted in dynamin diluting buffer: 6 mM Tris-HCl, 20 mM NaCl, and 0.02% Tween-80, pH 7.4) was pre-incubated with 10 µg/ml PS for 15 minutes. Following preincubation, dynamin was incubated in GTPase buffer (5 mM Tris-HCl, 10 mM NaCl, 2 mM Mg^{2+}, 0.05% Tween-80, pH 7.4, 1 µg/ml leupeptin, and 0.1 mM PMSF) and 0.3 mM GTP in the presence of test compound for 10 min for dynamin I and 90 min for dynamin II at 37^{o}C. The final assay volume was 150 µl in round-bottomed 96-well plates. Dynamin I and II were stimulated using 10 µg/ml PS liposomes to achieve normalised stimulation conditions. The reaction was terminated with 10 µl of 0.5 M EDTA, pH 8.0, and to each well 40µl of malachite green solution was added (2% (w/v) ammonium molybdite tetrahydrate, 0.15% (w/v) malachite green, and 4 M HCl; filtered through 0.45-µm filters, and stored in the dark up to 2 months at room temperature). Colour was developed for 5 min and the absorbance determined on a microplate spectrophotometer at 650 nm (VERSA$_{max}$ microplate reader, Molecular Device, Sunnyvale, CA). The IC_{50} values were calculated using Graphpad Prism v5, and data was

expressed as mean ± 95% confidence interval (CI) for triplicates within one experiment or mean ± SEM for 3 or more independent experiments.

***In vitro* growth inhibition (MTT assay)**

Growth inhibitory assays were carried out as described previously (2, 3). Cells in logarithmic growth were transferred to 96-well plates. Cytotoxicity was determined by plating cells in duplicate in 100 µl medium at a density of 2500 cells/well for HT29, H460, A431, and Du145 cells, 3000 cells/well for SW480, 3500 cells/well for MCF7, BE2-C and SJ-G2, and 2000 cells/well for A2780. On day 0, (24 h after plating) when the cells were in logarithmic growth, 100 µl medium with or without the test agent (drug) was added to each well. After 72 h drug exposure growth inhibitory effects were evaluated using the MTT (3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay and absorbance read at 540nm. GI_{50} values were calculated from the MTT dose response curve from three independent experiments, each performed in duplicate. The GI_{50} was the drug concentration at which cell growth is 50% inhibited based on the difference between the optical density values on day 0 and those at the end of drug exposure. The results were assessed by analysis of variance. Data were analysed using Statistica (Ver 4.5; Statsoft, Tulsa. Okla.)

Trypan blue exclusion

Cells were seeded in 10 cm^2 dishes ($1x10^5$ cells/dish). On day 0 (24 h after seeding), cells in triplicate were treated in the presence or absence of the dynamin inhibitor at concentrations of 1, 3, 10 and 30 µM. After 20 h, the cell number and viability were measured using a Vi-CELL XR cell viability analyser as previously described (4).

Lactate dehydrogenase (LDH) cytotoxicity

Cytotoxicity was assayed by determination of lactate dehydrogenase (LDH) activity. HeLa cells were seeded in 96 well plates. Asynchronously growing cells and cells

synchronised at G_2/M were treated in the presence or absence of the dynamin inhibitor at the indicated concentration for 8 h. The supernatant (50 µl) was added to 100 µl of LDH assay reagent (Sigma-Aldrich) and the reaction was allowed to developed for 20 min. Absorbance was measured at 490 nm and 690 nm (plate background absorbance). Values were normalised to drug/media background value and toxicity was calculated as a percentage of a control cell lysed with 20% Triton-X-100.

Colony-formation

Cells were seeded in 6-well plates (100 cells/well). On day 0 (24 h after seeding), cells in triplicate were treated in the presence or absence of the dynamin inhibitor at concentrations of 0.1, 0.3, 1, 3, and 10 µM. Cells were fixed in 10% formaldehyde/PBS after 7 days. The number of colonies containing ≥20 cells were counted per sample after staining with 0.2% crystal violet.

Cell cycle analysis by flow cytometry

Cells ($5x10^5$) were grown in 10 cm dishes. Following inhibitor treatment cells (floating and adherent) were collected and single-cell suspensions were fixed in 80% ice-cold ethanol at -20°C for at least 16 hours. Cells were stained with propidium iodide and the cell cycle was analysed as described previously (5). Cell cycle profiles were acquired with a FACS Canto Flow Cytometer (Becton Dickinson) using FACS Diva software (v5.0.1) at 488 nm. Cell cycle profiles were analysed using FlowJo software (v7.1).

Immunofluorescence microscopy

Cells were fixed in ice-cold 100% methanol for 10 min at -20ºC, and then blocked in 3% bovine serum albumin/PBS for 45 mins prior to application of a primary antibody:anti-CaN (AB-1; Calbiochem), anti-dynI phospho-S778 (6), anti-α-tubulin (clone DM1A; Sigma) and anti-γ-tubulin (GTU88; Sigma). Donkey anti-mouse Texas Red dye-conjugated AffiniPure

secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) were then applied. Cell nuclei were counterstained with DAPI (4', 6'-diamidino-2-phenylindole) (Sigma). Cells were washed three times with PBS between each step except for after blocking. Cells were viewed and scored for multinucleation with a fluorescence microscope (Olympus). Images were captured under an Olympus IX80 inverted microscope using a 100x oil immersion lenses and deconvolved using AutoDeblur v9.3 (AutoQuant Imaging, Watervliet, NY).

Immunoblotting

Cell lysates were prepared as described previously (7). In brief, cells were collected by centrifugation, washed with PBS, then resuspended in ice-cold lysis buffer (20 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and EDTA-free Complete protease inhibitor cocktail (Roche)) for 30 mins. The supernatant (cell lysate) was collected following centrifugation at 13,000 rpm for 30 min at 4ºC. Cell lysates (100 μg) were fractionated by SDS-PAGE for immunoblot analysis to detect cleaved PARP (Cell Signaling). Primary antibody was detected by incubation with horseradish peroxidise-conjugated anti-goat or anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Blotted proteins were visualised using the ECL detection system (Pierce).

References

1. Quan A, Robinson PJ. Rapid purification of native dynamin I and colorimetric GTPase assay. Methods Enzymol 2005;404:556-69.

2. Bergman AM, Ruiz vH, V, Veerman G, Kuiper CM, Peters GJ. Synergistic interaction between cisplatin and gemcitabine in vitro. Clin Cancer Res 1996;2:521-30.

3. Sakoff JA, Ackland SP. Thymidylate synthase inhibition induces S-phase arrest, biphasic mitochondrial alterations and caspase-dependent apoptosis in leukaemia cells. Cancer Chemother Pharmacol 2000;46:477-87.

4. Joshi S, Perera S, Gilbert J, Smith CM, Gordon CP, McCluskey A, et al. The dynamin inhibitors MiTMAB and OcTMAB induce cytokinesis failure and inhibit cell proliferation in human cancer cells. Mol Cancer Ther 2010;9:1995-2006.

5. Dix BR, O'Carroll SJ, Myers CJ, Edwards SJ, Braithwaite AW. Efficient induction of cell death by adenoviruses requires binding of E1B55k and p53. Cancer Res 2000;60:2666-72.

6. Anggono V, Smillie KJ, Graham ME, Valova VA, Cousin MA, Robinson PJ. Syndapin I is the phosphorylation-regulated dynamin I partner in synaptic vesicle endocytosis. Nat Neurosci 2006;9:752-60.

7. Fabbro M, Savage K, Hobson K, Deans AJ, Powell SN, McArthur GA, et al. BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage. J Biol Chem 2004;279:31251-8.

Supplementary Tables

Supplementary Table 1. IC_{50} values for the *in vitro* inhibition of dynamin I or dynamin II GTPase activity by the dynoles™.

Compound	DynI IC_{50} (μM)	DynII IC_{50} (μM) (Corrected against basal activity)	Selectivity ratio (DII/DI ratio)
Dynole™ 25	22.3 ± 11.1	20.1 ± 40.2	0.9 fold
Dynole™ 26	11.6 ± 7.4	17.9 ± 22.5	1.6 fold
Dynole™ 33	8.2 ± 1.7	27.4 ± 18.3	3.3 fold
Dynole™ 34-2	6.9 ± 1.0	14.2 ± 7.7	2.1 fold
Dynole™ 35	19.3 ± 4.0	41.9 ± 38.0	2.2 fold

Supplementary Figure Legends

Supplementary Figure 1. Chemical structure of dynoles™.

Supplementary Figure 2. The dynoles™ disrupt CaN localisation at the FMRs. **A,** G_2/M synchronised HeLa cells were treated with vehicle or the indicated dynamin inhibitor (10 μM). Cells were fixed after 2.5 h and stained for CaN, phospho-dynII^{S764} and γ-tubulin. The graph shows the percentage of cytokinetic cells that displayed the indicated proteins in correct ring localisation (mean ± S.D. from two independent experiments). **B,** Representative immunofluorescence images of CaN (green), phospho-dynII^{S764} (green) and γ-tubulin (red) localisation in HeLa cells treated with DMSO, MiTMAB, dynole™ 34-2 and dynole™ 35 as described in panel A. Treatment with the dynole™ dynamin inhibitors disrupts ring formation of CaN, but not phospho-dynII, at the FMRs. α-tubulin is shown in red in rows 2 and 3. DNA is shown in blue.