The Dynamin Inhibitors MiTMAB and OcTMAB Induce Cytokinesis Failure and Inhibit Cell Proliferation in Human Cancer Cells

Sanket Joshi1, Swetha Perera1, Jayne Gilbert2, Charlotte M. Smith1, Anna Mariana1, Christopher P. Gordon3, Jennette A. Sakoff2, Adam McCluskey3, Phillip J. Robinson1, Antony W. Braithwaite1,4, and Megan Chircop (nee Fabbro)1

Abstract

The endocytic protein dynamin II (dynII) participates in cell cycle progression and has roles in centrosome cohesion and cytokinesis. We have described a series of small-molecule inhibitors of dynamin [myristyl trimethyl ammonium bromides (MiTMAB)] that competitively interfere with the ability of dynamin to bind phospholipids and prevent receptor-mediated endocytosis. We now report that dynII functions specifically during the abscission phase of cytokinesis and that MiTMABs exclusively block this step in the cell cycle. Cells treated with MiTMABs (MiTMAB and octadecyltrimethyl ammonium bromide) and dyn-depleted cells remain connected via an intracellular bridge for a prolonged period with an intact midbody ring before membrane regression and binucleate formation. MiTMABs are the first compounds reported to exclusively block cytokinesis without affecting progression through any other stage of the cell cycle. Thus, MiTMABs represent a new class of antimiticotic compounds. We show that MiTMABs are potent inhibitors of cancer cell growth and have minimal effect on nontumorigenic fibroblast cells. Thus, MiTMABs have toxicity and antiproliferative properties that preferentially target cancer cells. This suggests that dynII may be a novel target for pharmacologic intervention for the treatment of cancer. Mol Cancer Ther; 9(7); 1995–2006. ©2010 AACR.

Introduction

Dynamin II (dynII) is a member of the dynamin superfamily, composed of three classic dynamins and four dynamin-related proteins conserved throughout eukaryotes (1). Among the three human dynamin genes, dynI is neuron specific, dynII is ubiquitously expressed, and dynIII is found in testis and brain (2). DynII is the ancestral form most closely related to the dynamin-related proteins. DynII is a 100-kDa GTPase enzyme best known for its role in membrane trafficking processes, specifically clathrin-mediated endocytosis (1, 3, 4). It also participates in caveola-mediated and clathrin-and caveola-independent endocytosis (5–8), macropinocytosis (9), phagocytosis (10, 11), and trafficking from the trans-Golgi network (12–14). Involvement of dynII in nonmembrane trafficking processes has been reported, including regulation of actin assembly and reorganization via interactions with actin-binding proteins (15–18). Whether dynII functions in these processes in an endocytic-independent or endocytic-dependent manner remains to be determined. DynII also participates in apoptosis. Overexpressed dynII activates caspase-3, triggering apoptosis in a p53-dependent manner (19), and this is dependent on its GTPase activity (19). Mutations in the GTPase effector domain that block dynII assembly enhance caspase-3 activation (20). DynII-induced apoptosis therefore seems to be independent of its endocytic function.

DynII also plays a role in cell cycle progression. During interphase, dynII localizes to centrosomes, participating in centrosome cohesion (21, 22). It is unknown if dynII is associated with the mitotic centrosome. DynII is also associated with the final stage of mitosis, cytokinesis (21, 23–26). During cytokinesis, dynamin localizes to the spindle midzone and the intracellular bridge (21, 26). DynII-knockout cells grow at a slower rate than their wild-type counterparts (24). These cells exhibit cytokinesis defects, whereby an increased percentage of cells is associated with the final stage of mitosis, cytokinesis (21, 23–26). During cytokinesis, dynamin localizes to the spindle midzone and the intracellular bridge (21, 26). DynII-knockout cells grow at a slower rate than their wild-type counterparts (24). These cells exhibit cytokinesis defects, whereby an increased percentage of cells is

www.aacrjournals.org
probing the cellular roles of dynamin (27–30). Dynasore inhibits dynamin GTPase function, which blocks clathrin-mediated endocytosis in nonneuronal cells and synaptic vesicle endocytosis in hippocampal neurons (31). We have reported a series of dimeric tyrphostins (Bis-Ts), long-chain amines and ammonium salts [collectively known as MiTMABs (myristyl trimethyl ammonium bromides)], and dynoles (28, 29, 32) as novel inhibitors of dynamin GTPase activity with a broad range of IC₅₀ values. The Bis-Ts inhibit dynamin GTPase activity in vitro. Their effect on endocytosis in cells has not yet been reported. MiTMABs inhibit dynamin GTPase activity by disrupting the PH (pleckstrin homology) domain-phospholipid interaction (28). Consequently, these are pan-dynamin inhibitors that also inhibit dynII-dependent receptor-mediated endocytosis in U2OS osteosarcoma cells and dynal-dependent synaptic vesicle endocytosis in neurons (32). Two of the most potent MiTMABs are myristyl trimethyl ammonium bromide (MiTMAB) and octadecyltrimethyl ammonium bromide (OctTMAB). Their effect on dynamin functions other than its role in endocytosis has not been investigated (33). Here, we aimed to determine the effect of these inhibitors on the cell cycle. We show that MiTMABs (collectively referring to MiTMAB and OcTMAB) are a new class of antimitotic compounds as they exclusively induce cytokinesis failure without affecting any other mitotic phase. This is consistent with a role for dynamin in cytokinesis. Growth arrest and cell death followed cytokinesis failure in cancer cells. Slow-growing nontumorigenic fibroblasts were less sensitive to cell death.

Materials and Methods

Cell culture
HeLa and H460 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. All other cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. All cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

Drugs
The active dynamin inhibitors MiTMAB (also known as tetradecyl trimethylammonium bromide, CAS no. 1119-97-7) and OcTMAB (CAS no. 1120-02-1; Sigma-Aldrich Co.) and the inactive analogue 2-(dimethylamino) ethyl myristate [2-(DiMA)EM; Lancaster Synthesis] were prepared as 30 mmol/L stock solutions in DMSO, stored at −20°C. Before further dilution in the assay, the drugs were diluted in 50% (v/v) DMSO/20 mmol/L Tris-HCl (pH 7.4) or media and stored at −20°C. The final concentration of DMSO in the GTPase assay is 1%.

Chemical synthesis of RO-3306
RO-3306 was synthesized in-house (Supplementary Fig. S1) using a previously reported protocol (34).

Cell synchronization and treatment with dynamin inhibitors
The selective cyclin-dependent kinase-1 (cdk1) small-molecule inhibitor RO-3306 (9 μmol/L; ref. 34) accumulated cells at the G2-M boundary followed by synchronous mitotic progression after RO-3306 washout, as previously reported (35). When cells were transfected with small interfering RNA (siRNA), RO-3306 was added 54 hours after transfection. Immediately following RO-3306 removal, cells were treated with a dynamin inhibitor or the Aurora kinase inhibitor ZM447439 (Tocris Bioscience). As a control, cells were released in drug-free medium, 0.1% DMSO, or negative analogue 2-(DiMA)EM.

Protein production
Native dyn1 was extracted and purified from the peripheral membrane fraction of whole sheep brain (36) by affinity purification on GST-AmphII-SH3-Sepharose as described previously (37). The yield of dyn1 protein from 250-mg sheep brain is 8 to 10 mg. Recombinant dyn1 produced from insect cells (Sf9) was kindly provided by Sandra L. Schmid (The Scripps Research Institute, San Diego, CA).

Malachite green GTPase assay
The malachite green GTPase assay is a colorimetric based assay, where malachite green dye was used for the sensitive colorimetric detection of orthophosphate (Pi). The assay procedure has been described in detail previously and is based on stimulation of purified dynamin protein by sonicated phosphatidylserine liposomes (37).

Purified dyn1 (7 nmol/L) or dynII (20 nmol/L; diluted in dynamin diluting buffer [6 mmol/L Tris-HCl, 20 mmol/L NaCl, and 0.02% Tween 80 (pH 7.4)] was incubated in GTPase buffer [5 mmol/L Tris-HCl, 10 mmol/L NaCl, 2 mmol/L Mg²⁺, 0.05% Tween 80 (pH 7.4), 1 μg/mL leupeptin, and 0.1 mmol/L phenylmethylsulfonyl fluoride] and 0.3 mmol/L GTP in the presence of test compound for 30 minutes at 37°C. The final assay volume was 150 μL. The assay was conducted in round-bottomed 96-well plates. Incubations of the plates were done in dry heating block with shaking at 600 rpm (Eppendorf Thermomixer). Dynamin GTPase activity was maximally stimulated by addition of different concentrations of phosphatidylserine liposomes. In these assays, dyn1 and dynII were stimulated using 2 and 20 μg/mL phosphatidylserine liposomes, respectively. The reaction was terminated with 10 μL of 0.5 mol/L EDTA (pH 8.0), and the samples were stable at room temperature for several hours. To each well, 40 μL of malachite green solution were added [2% (w/v) ammonium molybdate tetrahydrate, 0.15% (w/v) malachite green, and 4 mol/L HCl]; the solution was passed through 0.45-μm filters and was stored in the dark for up to 2 months at room temperature. Color was allowed to develop for 5 minutes (stable for up to 2 hours), and the
absorbance of each sample was determined on a microplate spectrophotometer at 650 nm (VERSAmax microplate reader, Molecular Devices). The IC\textsubscript{50} values were calculated using GraphPad Prism v5, and data were expressed as mean ± 95% confidence interval of triplicates within one experiment.

**MTT assay**

Growth inhibitory assays were carried out as described previously (38, 39). Cells in logarithmic growth were transferred to 96-well plates (100 μl medium per well) at a density of 2,500 cells per well for HeLa, HT29, H460, A431, and DU145; 3,000 cells per well for SW480; 3,500 cells per well for MCF-7, BE2-C, and SJ-G2; and 2,000 cells per well for A2780. On day 0 (24 hours after plating), cells in duplicate were treated with or without the dynamin inhibitor. After 72 hours, drug exposure, cytotoxicity, and growth inhibitory effects were evaluated using the MTT assay. GI\textsubscript{50} values were calculated as mean ± 95% confidence interval of triplicates within one experiment.

**Colony formation assay**

Cells were seeded in six-well plates (100 cells per well). On day 0 (24 hours 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 μmol/L. Cells were fixed in 10% formaldehyde/PBS after 7 days (HeLa and H460 cells) or 10 days (SW480 cells). The number of colonies containing ≥20 cells was counted per sample after staining with 0.2% crystal violet.

**Cell growth and drug washout assay**

Cells were seeded in 10-cm dishes (1 × 10\textsuperscript{5} cells per dish). On day 0 (24 hours after seeding), cells in triplicate were treated in the presence or absence of the dynamin inhibitor at concentrations of 1, 3, and 10 μmol/L. Cell number and viability were measured using a Vi-CELL XR cell viability analyzer. Excess drug was removed from the viable cells by washing three times with drug-free medium. Viable cells were seeded in 10-cm dishes and incubated at 37°C in a humidified 5% CO\textsubscript{2} atmosphere. Cell number and viability were reassessed after 6 days.

**Cell cycle analysis by flow cytometry**

Cells (5 × 10\textsuperscript{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 analyzed as described previously (40). Cell cycle profiles were acquired with a FACSCanto flow cytometer (Becton Dickinson) using FACSDiva software (v5.0.1) at 488 nm. Cell cycle profiles were analyzed using FlowJo software (v7.1).

### Immunofluorescence microscopy

Cells were fixed in ice-cold 100% methanol, and immunostaining was carried out as described previously (35) using the anti-α-tubulin (clone DM1A, Sigma) and anti-γ-tubulin (Sigma) antibodies. Cells were viewed and scored with a fluorescence microscope (Olympus BX51). Fluorescence images were captured and processed using an Olympus IX80 inverted microscope using 40× or 100× oil immersion lenses and Metamorph software. Images were deconvolved using AutoDeblur v9.3 (AutoQuant Imaging).

#### Time-lapse microscopy

HeLa and H460 cells (1 × 10\textsuperscript{5} per well) were seeded into six-well plates. Immediately following the release into the cell cycle following synchronization, cells were treated with the indicated molecule and viewed with an Olympus IX80 inverted microscope. A time-lapse series was acquired using a fully motorized stage, 10× objective, and Metamorph software using the time-lapse modules. Temperature was controlled at 37°C using the Incubator XL, providing a humidified atmosphere with 5% CO\textsubscript{2}. Images were captured every 10 minutes for 20 hours.

#### siRNA oligonucleotides

To knock down dynamin protein expression, Stealth Select 3 siRNA duplexes specifically targeting dynamin (Invitrogen) were used. Sequences of the siRNAs targeting dynamin are as follows: dynamin siRNA 1, 5′-AAUGUCGAUCAGCAGAAGAAUCUGG; dynamin siRNA 2, 5′-UCUCCUGCCGACUCAUAAGAC-3′; dynamin siRNA 3, 5′-AUUCACGCUUCU-GCAUGUGGC-3′. Stealth siRNA Negative Control Medium GC Duplex was used as a negative control (Invitrogen).

#### Cell transfection

For siRNA analysis, cells were seeded at 50% to 60% confluence and transfected with 1,000 pmol siRNA (per 10-cm dish for immunoblotting) or with 200 pmol siRNA (per well of a six-well plate for immunofluorescence and time-lapse microscopy experiments) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

#### Immunoblotting

Cell lysates were prepared as described previously (41). In brief, cells were collected by centrifugation, washed with PBS, then resuspended in ice-cold lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, and EDTA-free Complete protease inhibitor cocktail (Roche)] for 30 minutes. The supernatant (cell lysate) was collected following centrifugation at 13,000 rpm for 30 minutes at 4°C. Cell lysates (50 μg) were fractionated by SDS-PAGE for immunoblot analysis to detect dynII (Santa Cruz) and α-tubulin (Sigma).
antibody was detected by incubation with horseradish peroxidase–conjugated antigoat or antimouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Blotted proteins were visualized using the enhanced chemiluminescence detection system (Pierce).

### Results

#### MiTMAB and OcTMAB reduce cell proliferation and viability

Dynamin seems to participate in two roles during cell cycle progression. These include a role at the centrosome during interphase and at the midbody during cytokinesis to complete a mitotic division (21, 23–26). Therefore, we asked whether inhibition of dynamin by the small-molecule inhibitors MiTMAB and OcTMAB would affect cell proliferation and cell viability. The in vitro IC₅₀ values of MiTMAB and OcTMAB for dynI GTPase activity are 2.26 ± 0.53 and 0.92 ± 0.13 μmol/L, respectively (ref. 32; Table 1). MiTMAB and OcTMAB also inhibit dynII GTPase activity with IC₅₀ values of 8.4 ± 5.79 and 4.35 ± 2.42 μmol/L, respectively (Table 1). A third compound in the MiTMAB series is the ester derivative of myristic acid, 2-(DiMA)EM. It is important to note that although it inhibits the activity of purified dynamin in vitro (Table 1), it does not inhibit dynamin-mediated endocytosis in cells (32). This is because it is a prodrug that is rapidly cleaved to myristic acid (which is inactive) and dimethylamino ethanol by endogenous intracellular esterases (32). Therefore, 2-DiMA(EM) was used as a negative control throughout our study. Whereas dynI in

<table>
<thead>
<tr>
<th>IC₅₀ (μmol/L)</th>
<th>DynI</th>
<th>DynII</th>
<th>DynII/dynI selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>OcTMAB</td>
<td>0.92 ± 0.13</td>
<td>4.4 ± 2.4</td>
<td>4.7</td>
</tr>
<tr>
<td>MiTMAB</td>
<td>2.26 ± 0.53</td>
<td>8.4 ± 5.8</td>
<td>3.7</td>
</tr>
<tr>
<td>2-(DiMA)EM</td>
<td>2.49 ± 0.66</td>
<td>19.95 ± 28</td>
<td>8</td>
</tr>
</tbody>
</table>

NOTE: The in vitro GTPase activities of dynI and dynII were determined in the presence of increasing concentrations of MiTMABs and 2-(DiMA)EM. The table shows IC₅₀ values represented as mean (triplicates) with 95% confidence interval during a single experiment. Results are representative of two independent experiments.

Figure 1. Dynamin inhibitors MiTMAB and OcTMAB reduce proliferation and viability of cancer cells. A, cell proliferation was assessed by the MTT cytotoxicity assay in the presence of various concentrations of MiTMAB or OcTMAB after 72 h of continuous exposure. The values graphed are from three independent experiments, each carried out in duplicate. B, HeLa, H460, and SW480 cancer cells were treated with various concentrations (0.1, 0.3, 1, 3, and 10 μmol/L) of MiTMAB, OcTMAB, or the inactive analogue, 2-(DiMA)EM for 7 d. Line graphs show the relative percentage of colonies present at day 7. Values were normalized to untreated controls to equal 100%.
Dynamin Inhibitors Cause Cytokinesis Failure

this study was the endogenous form purified from sheep brain, dynII was produced as a recombinant protein expressed in Sf9 insect cells. Subtle differences between the dynI and dynII assay conditions mean that the relative IC50 data are indicative only, with differing dynamin and lipid concentrations being required for each assay. Therefore, we do not consider the 4- to 8-fold differences in DynII/I selectivity ratio as indicating that the compounds have any selectivity between the two dynamins (Table 1). Throughout the article, MiTMABs refer to both MiTMAB and OcTMAB. We first assessed the growth inhibitory effect of MiTMABs on 10 cancer cell lines derived from different tissues: HT29 and SW480 (colon), HeLa (cervical), MCF-7 (breast), A2780 (ovary), H460 (lung), A431 (skin), DU145 (prostate), BE(2)-C (neuronal), and SJ-G2 (glial). After 72 hours of continuous exposure to the dynamin inhibitors, all cell lines showed a dose-dependent decline in cell growth following treatment with MiTMABs (Fig. 1A). At concentrations >5 μmol/L, cell death was evident as the response curves decreased below the zero mark, indicating that the number of viable cells at the end of the experiment was less than the number at the start. At concentrations below 5 μmol/L, growth inhibition is likely a combination of growth arrest and cell death. The GI50 values and dose-response curves (Table 2) reveal that the sensitivity to MiTMABs varied among the cell lines, with A431, HeLa, and HT29 cells being the most sensitive and DU145 and H460 cells being the least sensitive to the dynamin inhibitors. Although OcTMAB showed consistently lower GI50 values than MiTMAB, the potency of growth inhibition was comparable.

A colony formation assay confirmed the antiproliferative effect of MiTMABs in HeLa, H460, and SW480 cells with a 50% reduction in colony formation at ∼0.5 μmol/L (Fig. 1B). In contrast, 2-(DiMA)EM caused a 50% reduction in colony formation at >10 μmol/L (Fig. 1B). Therefore, the dynamin inhibitors MiTMAB and OcTMAB inhibit cell proliferation and reduce viability in a range of cancer cells.

**MiTMABs cause cytokinesis failure**

We next determined if the antiproliferative and cytotoxic effects of MiTMABs were due to their ability to inhibit cell cycle progression in a cell cycle stage-dependent manner. First, the cell cycle profile of asynchronously growing HeLa cells was assessed using flow cytometry following a 48-hour exposure to the drugs. Treatment with MiTMABs caused marked cell cycle alterations (Fig. 2A). Consistent with their effect on cell viability, they increased the sub-G1 (<2N DNA content) population, indicating apoptosis. G1 and S phase populations decreased, whereas the population of cells containing 4N DNA content (G2-M) increased. The accumulation of cells containing 4N DNA content following MiTMAB and OcTMAB treatment was confirmed in HeLa, H460, and SW480 cells following synchronization at the G2-M border using the cdk1 inhibitor RO-3306 (Fig. 2B; ref. 34). Successful synchronization with RO-3306 was confirmed (Supplementary Fig. S2). Therefore, MiTMABs disrupt cell cycle progression.

The accumulated cell population with 4N DNA content may be multinucleated rather than arrested in G2 or M phase as suggested by the reported role of dynII in cytokinesis (24). To confirm this hypothesis, we carried out immunofluorescence microscopy analysis. Indeed, an increase in multinucleation was observed in MiTMAB- and OcTMAB-treated HeLa cells (Fig. 2C and D), indicating failed cytokinesis. Cytokinesis failure was also evident in H460 cells, as a large proportion of these cells were connected via an intracellular bridge following exposure to MiTMABs (Fig. 2C and D). A short intracellular bridge located in the middle of the two daughter cells (Fig. 2D, bottom right) was observed on occasion. However, the intracellular bridge of most H460 cells treated with MiTMABs differed from that of control cells. It was often extended in length (Fig. 2D, top right) or off-center and curved in appearance (Fig. 2D, bottom left). Cytokinesis failure was also observed in these cells following exposure to the Aurora kinase inhibitor ZM447439 (Fig. 2C). MiTMAB also induced multinucleation in asynchronously growing HeLa cells (Supplementary Fig. S3). Thus, the synchronization agent RO-3306 had no effect. The percent multinucleated HeLa cells increased with longer treatment periods of MiTMAB because additional cells were able to undergo mitosis. These findings show that MiTMAB and OcTMAB cause cytokinesis failure.

**Cytokinesis failure is at the membrane abscission step**

Cytokinesis in animal cells requires two steps: (a) membrane ingestion and (b) membrane abscission.
Evidence supports a role for dynII in the abscission step (24). Therefore, we sought to determine if the dynamin inhibitors disrupt the abscission step. We used time-lapse microscopy to observe HeLa cells progressing through mitosis. By analyzing only those cells that entered mitosis, we found that more than 30% of cells treated with 10 μmol/L OcTMAB failed cytokinesis and became multinucleated, whereas almost 100% of cells failed cytokinesis at 30 μmol/L (Fig. 3B, left). Comparable results were obtained following exposure to MiTMAB. In contrast, the inactive analogue 2-(DiMA) EM had negligible effect on cytokinesis even at
concentrations as high as 100 μmol/L (Fig. 3B, left). MiTMAB- and OcTMAB-treated HeLa cells take approximately 25 to 32 minutes longer to complete mitosis (Fig. 3B, right; Supplementary Fig. S4A and Movie S1). Therefore, we calculated the time cells took to undergo the following three mitotic transitions to identify the point of failure: (a) prophase (Pro) to metaphase (Met), (b) Met to full membrane ingression (Ing), and (c) Ing to generation of either two daughter cells (Comp) or a multinucleated cell (Multi; Fig. 3A). Drug-treated cells completed the Pro-Met and Met-Ing transitions with identical kinetics to control cells. Thus, mitotic entry, chromosome alignment, and segregation were unaffected. MiTMAB- or OcTMAB-treated cells spent twice the amount of time in the Ing-Comp or Multi transition than control cells (Fig. 3B, right). Thus, cleavage furrow formation and membrane ingression occurred normally in MiTMAB- or OcTMAB-treated cells, but remained connected via an intracellular bridge for a prolonged period of time before membrane regression, forming a multinucleated cell (Supplementary Fig. S4A). These cells contained an intact midbody ring because γ-tubulin was present at the center of the intracellular bridge (Fig. 3C). A similar cytokinesis failure phenotype was induced by MiTMAB and OcTMAB in H460 cells (Fig. 3D; Supplementary Fig. S4B and Movie S2). H460 cells were more sensitive to dynamin inhibition than HeLa cells. For example, exposure to 10 μmol/L MiTMAB resulted in approximately 8% of HeLa cells compared with more than 60% of H460 cells failing cytokinesis (Fig. 3B and D). In addition, unlike HeLa cells, H460 cells did not become multinucleated during the 20-hour experimental period but remained connected via an intracellular bridge (Supplementary Fig. S4B). This is consistent with our immunofluorescence microscopy analysis of fixed cells (Fig. 2C). By 72 hours, however, the percentage of MiTMAB- and OcTMAB-treated H460 cells connected via an intracellular bridge was not significantly different than control cells [untreated, 0.1% DMSO, and 2-(DIMA)EM] and the presence of multinucleated H460 cells was now apparent (Supplementary Fig. S5). Therefore, MiTMAB and OcTMAB cause cytokinesis failure by disrupting the abscission step.

**DynII depletion leads to cytokinesis failure at the abscission stage**

To determine if the cytokinesis failure phenotype caused by MiTMABs is consistent with dynII being the drug target, we analyzed cells depleted of dynamin using siRNA. Three different siRNA molecules depleted dynII levels in HeLa cells by 75% to 95% (Fig. 4A). In an analogous manner to treatment with MiTMABs, a significant number of dynamin-depleted cells were multinucleated (Fig. 4B), indicating failed cytokinesis. This supports and extends previous invertebrate studies (25) that dynamin is required for successful completion of mitosis. Analysis of dynamin-depleted HeLa cells by time-lapse microscopy revealed an identical cytokinesis failure phenotype to cells treated with MiTMABs. Dynamin-depleted HeLa cells exclusively affected cytokinesis, failing at the abscission step, as evident by an increased period of time spent connected via an intracellular bridge before the generation of either a multinucleated cell or two independent cells (Ing-comp or multi; Fig. 4C). No cytokinesis defects were observed in cells treated with control luciferase (Luc) siRNA (Fig. 4C). These findings reveal that dynII is involved in the abscission step of cytokinesis in HeLa cells.

**Viability and proliferation of nontumorigenic fibroblasts**

Our findings indicate that the dynamin inhibitors have in vitro anticancer properties analogous to those of other small-molecule inhibitors targeting the mitotic proteins, Aurora kinases, Plk, and KSP (42). Some of these inhibitors are in preclinical and clinical trials for the treatment of cancer (42). To assess if inhibition of dynII is a novel potential approach for cancer treatment, we sought to determine the effect of MiTMAB and OcTMAB on the viability and proliferation of nontumorigenic fibroblast cells after 7 days of continuous exposure and for a further 6 days after drug washout. Because the skin carcinoma cell line A431 was the most sensitive to MiTMAB- and OcTMAB-induced growth inhibition, we chose to examine the effect of these compounds in the primary skin fibroblast cell lines F-1, Fre119S(1CF2), and F80/10-3R (hTERT-immortalized fibroblasts) and in NIH3T3 fibroblast cells. F-1 and F80/10-3R cells are very slow growing with a cell doubling time of >10 days and 3 to 4 days, respectively, compared with ~24 hours for A431 cells. However, Fre119S and NIH3T3 cells have a doubling rate similar to that of A431 cells. As this was a sequential experiment, the MTT assay was not appropriate and total cell number was used to more appropriately assess regrowth after drug removal. MiTMAB and OcTMAB

---

**Figure 2.** Dynamin inhibitors MiTMAB and OcTMAB cause cytokinesis failure. A, Asynchronously growing HeLa cells were treated for 48 h with MiTMAB, OcTMAB, or controls [untreated, 0.1% DMSO, and 2-(DIMA)EM]. DNA content was assessed by flow cytometric analysis of cells labeled with propidium iodide. The graph shows the percentage of cells in each cell cycle phase. B, HeLa, H460 (top), and SW480 (bottom) cells were synchronized at the G_{2}-M boundary using RO-3306 and subsequently released into either drug-free medium or medium containing the dynamin inhibitor MiTMAB or OcTMAB or controls. Cells were labeled with propidium iodide and analyzed by flow cytometry at the indicated time following release. Graphs illustrate the percentage of cells with 4N DNA content. C, cells were synchronized and treated as described in B, but were also treated with the Aurora kinase inhibitor ZM447439. At 6 h post-release from RO-3306, cells were fixed and stained for α-tubulin, and the percentage of cells that were multinucleated (HeLa) or connected via an intracellular bridge (H460) was scored using immunofluorescence microscopy. Columns, mean from three independent experiments; bars, SEM. D, representative microscopy images of data described in C illustrating multinucleated HeLa cells and H460 cells connected via an intracellular bridge following treatment with MiTMAB. Red, α-tubulin; blue, DNA. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test).
Figure 3. Dynamin inhibition blocks cell cycle progression specifically at abscission. A, schematic diagram of the phases in mitosis. Pro, prophase; Met, metaphase; Ana, anaphase; Tel, telophase; Ing, complete furrow ingression; Comp, completion; Multi, multinucleation; Con, cells remaining connected with the intracellular bridge. B, HeLa cells were synchronized at the G2-M boundary and treated with MiTMAB, OcTMAB, or controls as described in the legend to Fig. 2B. Cells were visualized by time-lapse microscopy and those cells that entered mitosis were scored for failed cytokinesis (left) and the time taken to progress through mitosis (right). n > 160 cells per sample. C, representative microscopy images of an untreated HeLa cell and a HeLa cell treated with 10 μmol/L MiTMAB (as described in B) showing that dynamin inhibitors do not disrupt the centrally located midbody ring as indicated by γ-tubulin (green). D, H460 cells were synchronized, treated, and analyzed as described in B. n > 50 cells per sample. Columns, mean for each experimental condition; bars, SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test).
inhibited the cell growth of all four fibroblast cell lines (Fig. 5), comparable with the cancer-derived cell lines. However, unlike the cancer cells, a trypan blue assay revealed that a significant number of fibroblast cells were viable after 7 days of exposure to MiTMABs (data not shown). Following drug washout, proliferation of these fibroblast cells was restored within the next 6 days. This was observed for all concentrations of MiTMABs tested in all four fibroblast cell lines, except for F80/10-3R cells at a concentration of 10 μmol/L (Fig. 5). Thus, nontumorigenic fibroblasts seem to be less sensitive to MiTMABs than cancer cells.

Discussion

A role for dynII in cytokinesis has been previously suggested, and tentative links suggest that it participates in the abscission phase (21, 23–26). With the availability of small-molecule inhibitors of dynamin, we set out to dissect the role of dynamin in cytokinesis and determine the effect of these inhibitors on cell viability and proliferation. We found that the dynamin inhibitors MiTMAB and OcTMAB are potent antiproliferative compounds, reducing cell viability and growth in all 10 cancer cell lines analyzed with a range in sensitivity. We reveal that the antiproliferative properties of MiTMAB and OcTMAB are due to their ability to block cytokinesis, specifically at the abscission phase. Therefore, MiTMABs are a new class of antimitotic compounds with specificity for cytokinesis exclusively.

The results show that dynII function is associated with the second phase of cytokinesis, abscission, and not the first phase, membrane ingression. Dynamin depletion by siRNA did not affect the ability of cells to form a cleavage furrow but rather prolonged the time cells spent connected via an intracellular bridge. This is consistent with recently published data that reported an increased frequency of intracellular bridge connections in dynII-knockout mouse embryonic fibroblasts (24). Following a delay at the intracellular bridge stage, dyn-depleted cells eventually resulted in membrane regression and the formation of binucleated cells.

Our findings indicate that the phenotypes induced by MiTMAB and OcTMAB are due to targeted inhibition of dynamin because they induced an analogous cytokinesis failure phenotype in dynamin-depleted cells. The response was highly specific, as no other defects were observed in cells progressing through any other mitotic stage. This strongly indicates that these small molecules are likely to function in cells primarily by inhibiting dynamin. The cytokinetic time delay induced by MiTMAB differed between cell lines. For example, H460 cells were connected via an intracellular bridge for a minimum of 20 hours before multinucleate formation, compared with only 20 to 30 minutes in HeLa cells. The reason underlying this difference is unknown. Nevertheless, MiTMAB and OcTMAB induce cytokinesis failure by preventing membrane abscission.

Figure 4. Dynamin depletion causes cytokinesis failure. A, HeLa cells were either untransfected or transfected with a negative (Neg.) siRNA control or three dynamin-specific siRNAs. At 96 h post-transfection, lysates (100 μg) were immunoblotted with an anti-dynII antibody. An immunoblot of α-tubulin revealed that protein loading in each sample was equivalent. B, quantitative analysis of multinucleation. Cells were transfected as described in A, then fixed at 120 h post-transfection for immunostaining with anti-α-tubulin antibodies. Per sample, >200 cells were scored for the presence of ≥2 nuclei per cell (multinucleation). Columns, mean for each experimental condition from at least three independent experiments; bars, SEM. C, dynamin-depleted cells prevent and/or delay the abscission stage of cytokinesis. HeLa cells were either untransfected or transfected with a negative (Neg.) siRNA control or dynamin siRNA 3. At 48 h post-transfection, cells were synchronized at the G2-M transition by the cdk1 inhibitor RO-3306 and then visualized by time-lapse microscopy. Cells were scored for the time taken to undergo mitosis (n > 120 cells per sample) as described in the legend to Fig. 3B. The graph illustrates that depletion of dynamin prolongs the mitotic phase compared with untreated or negative siRNA controls. Specifically, following chromosome segregation and completion of membrane ingression, the abscission stage is prolonged. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test).
Following cytokinesis failure, the cellular response to the dynamin inhibitors was cell growth arrest and cell death. This is consistent with targeted inhibition of dynamin, as dynII-knockout cells have been reported to grow at a slower rate than their wild-type counterparts (24). Although all cancer cell lines were essentially equally sensitive to MiTMAB and OcTMAB with regard to cell growth arrest, their effects on cell death differed. This perhaps reflects the underlying differences in their genetic aberrations. Nevertheless, the effect of these dynamin inhibitors on nontumorigenic cell growth is suggestive of tumor selectivity. The dynamin inhibitors are clearly targeting cytokinesis. In support of this idea, after 7 days of treatment with MiTMAB and OcTMAB, sufficient numbers of fibroblast cells, but not cancer cells, were viable and their proliferation was restored after MiTMAB washout. We have previously shown that washout of MiTMABs can restore dynamin-dependent receptor-mediated endocytosis in COS7 cells following a short 15-minute exposure to the drug (32). This reversible characteristic makes these small molecules a very powerful research tool for studying the functional role of dynII during cytokinesis.

Several inhibitors of mitotic proteins have emerged in the preclinical or early clinical development for the treatment of cancers (42). The target proteins to date have been cyclin-dependent kinase, checkpoint kinase, Aurora kinase, and Plk, KSP, and centromeric protein E. Inhibitors of these proteins have been evaluated in various hematologic and solid malignancies. Several findings suggest that dynamin is a new target in the development of inhibitors for pharmacologic intervention for the treatment of cancer: (a) Dynamin inhibitors, MiTMAB and OcTMAB, cause cytokinesis failure at the

**Figure 5.** Nontumor fibroblasts are less sensitive to dynamin inhibitor–induced cell death. Columns, mean total number of viable F-1, F80/10-3R, NIH3T3, and Fre119S(1CF2) cells present after 7 d of exposure to the dynamin inhibitors and after another 6 d following their washout; bars, SD. Removal of dynamin inhibitors restores proliferation of fibroblasts.
point of abscission followed by cell cycle arrest and cell death in human cancer cells (this study). No other reported antimitotic inhibitor targets this stage of cytokinesis exclusively. Thus, dynamin inhibitors represent a new class of antimitotic agents with a distinctly different mechanism of action. (b) Nontumorigenic fibroblasts are less sensitive to cell death induced by MiTMAB and OcTMAB than tumor cell lines (this study), suggesting that they may have a relative specificity for cancer cells. In addition, fibroblast cell proliferation can be restored following their removal (this study). Collectively, our findings establish a role for dynamin in the abscission phase of cytokinesis. Furthermore, we provide a rationale for the further investigation of dynamin inhibitors in oncology, with the possibility that such compounds will not only inhibit cell proliferation but will also induce tumor cell death, with the potential of causing tumor regression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Maggie Pai Chi Ma and Christine Smyth for their technical assistance.

Grant Support

National Health and Medical Research Council (NH&MRC) of Australia (P.J. Robinson and M. Chircop), the New South Wales Cancer Council (M. Chircop and J.A. Sakoff), and the NH&MRC Career Development Award (M. Chircop). A.W. Braithwaite is supported by Leader’s Fellowship from the Cancer Institute, New South Wales.

Received 09/22/2009; revised 04/19/2010; accepted 04/30/2010; published OnlineFirst 06/22/2010.

References

are surface-active small molecule dynamin inhibitors that block endocytosis mediated by dynamin I or dynamin II. Mol Pharmacol 2007;72:1425–39.
The Dynamin Inhibitors MiTMAB and OcTMAB Induce Cytokinesis Failure and Inhibit Cell Proliferation in Human Cancer Cells

Sanket Joshi, Swetha Perera, Jayne Gilbert, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-0161

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2010/06/22/1535-7163.MCT-10-0161.DC1

Cited articles
This article cites 42 articles, 17 of which you can access for free at:
http://mct.aacrjournals.org/content/9/7/1995.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/9/7/1995.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.