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Characterization of Agonist-Induced Motilin Receptor Trafficking and its Implications for Tachyphylaxis

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Abbreviations: MR, motilin receptor; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; SD, standard deviation.

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ABSTRACT

The motilin receptor (MR) is a member of the seven-transmembrane receptor family and is expressed throughout the gastrointestinal tract of humans and other species. Motilin, the natural MR peptide ligand, has profound stimulatory effects on gastrointestinal contractility, indicating a therapeutic potential of MR modulators. However, long-term clinical use of certain MR agonists is limited by tachyphylaxis, a reduced responsiveness to repeated compound exposure. The aim of this study was to characterize the ligand-induced endocytosis of MR, and test whether receptor trafficking contributes to tachyphylaxis. A cell-based assay was developed by fusing a green fluorescent protein (GFP) moiety to the motilin receptor, and high-content biology instrumentation was used to quantify time- and dose-dependence of MR-GFP endocytosis. Maximal internalization of MR-GFP was induced after 45 minutes of constant exposure to 80 nM motilin. This process was disrupted by nocodazole, suggesting an essential role for microtubules. Internalized MR-GFP vesicles disappeared within 15-45 minutes of motilin withdrawal but did not overlap with the lysosomal compartment, indicating that MR-GFP escaped degradation and was recycled back to the plasma membrane. Importantly, the kinetics of MR-GFP redistribution varied substantially when stimulated with motilin, erythromycin, ABT-229 or BMS-591348 at equipotent doses for Ca^{2+}-mobilization. Retardation of the intracellular MR-GFP sorting cycle appeared to correlate with the tachyphylaxis-inducing properties of each compound, but not its EC_{50}. These results indicate that MR internalization, de- and re-sensitization are ligand dependent, and that appropriate screening

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strategies may enable the development of small molecule agonists with ideal combinations of these distinct properties.

INTRODUCTION

Gastrointestinal (GI) motility disorders are of increasing importance in industrialized countries, as indicated by the rapidly growing number of patients suffering from diabetic gastroparesis, irritable bowel syndrome, gastroesophageal reflux disease, and chronic constipation. Current and future therapies aim at modulating gut smooth muscle contractions to alleviate the symptoms of GI motility disorders (Chovet, 2000). Motilin, a highly conserved peptide hormone expressed in the small intestine of humans and other species, is involved in the regulation of gastrointestinal motility. Mature motilin consists of the first 22 N-terminal amino acids of the inactive precursor protein. The stimulatory effects on GI smooth muscle contraction and gastric emptying of this important peptide are well documented and have attracted considerable attention to its therapeutic potential (Vantrappen and Peeters, 1989).

The effects of motilin are mediated through the recently identified motilin receptor (MR; originally termed GPR38 (Feighner et al. 1999)), which belongs to the superfamily of G protein-coupled or seven-transmembrane receptors (Pierce et al., 2002). GPR38 had been cloned based on its homology (53% amino acid identity) to the human growth hormone secretagogue receptor (GSHR), and was classified as an orphan GPCR because its natural ligand remained unknown

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(McKee et al., 1997). Screening of peptide and small molecule libraries against GPR38 using a functional signaling assay led to the identification of motilin as its cognate binding partner (Feighner et al. 1999). Interestingly, the antibiotic compound erythromycin was also shown to exhibit agonist activity, confirming earlier findings that its prokinetic effects on GI motility were mediated through the motilin receptor (Peeters et al., 1989). While erythromycin can be prescribed in the clinic to stimulate GI motility, its side-effect profile (vomiting, nausea, diarrhea) and antibiotic activity prevent long-term use. This has initiated a search for compounds with motilin receptor agonist activity that lack antibiotic effects and are better tolerated than erythromycin. Several potent erythromycin derivatives have been developed, including ABT-229 (Lartey et al., 1995) and GM-611 (Peeters, 2001). Patients treated with ABT-229 in clinical trials, however, experienced a decrease in response after repeated drug exposure (Talley et al., 2000; Netzer et al., 2002). It was proposed that motilin receptor desensitization, also termed tachyphylaxis, may have contributed to clinical failure (Tack and Peters, 2001), and recent publications appear to support this hypothesis based on agonist-induced Ca^{2+} measurements in cell culture experiments (Li et al., 2004; Thielemans et al., 2005).

Most G-protein-coupled receptors (GPCRs) undergo internalization upon agonist treatment (Pierce et al., 2002; Ferguson 2001). Once internalized, the receptor may be subject to three different fates; recycled back to the cell surface, targeted to the lysosomes for degradation, or retained within the endosomal compartment (Ferguson, 2001). The balance between these three fates along

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with the rate of de novo synthesis of new receptors determines the kinetics of desensitization and resensitization. Consequently, it is critical to investigate and understand the endocytosis patterns of a GPCR drug target in order to understand how tachyphylaxis arises. This information can then be utilized to guide the design of agonists that minimize desensitization.

Little is known about the intracellular fate of the motilin receptor at the molecular level upon agonist stimulation. For instance, we lack information on its endocytosis pattern and whether MR is subsequently degraded or recycled back to the plasma membrane. Most importantly, with respect to design of optimal modulators, it is unknown whether the properties of the ligand can influence any aspect of internalization, recycling, degradation or retention. Therefore, the aim of this study was to characterize the trafficking pattern of the motilin receptor inside the cell and relate these results to the functional tachyphylaxis-inducing properties of different agonists. To address these questions a stable cell line expressing a motilin-receptor GFP fusion protein was generated. The ligand-induced redistribution of MR-GFP was analyzed quantitatively by using fluorescence confocal microscopy in combination with high-content screening algorithms. The results indicate that receptor internalization is dependent on agonist dose and exposure time, and provide a critical role for microtubules. Furthermore, internalized MR-GFP molecules did not co-localize with lysosomes, suggesting that they were redistributed back to the cell surface and may be available for repeated signaling and internalization. Finally, the tachyphylaxis-inducing properties of different MR agonists were found to correlate with their

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propensity to induce receptor internalization, but not with their signaling potency (EC_{50}). Not only does this novel quantitative internalization assay provide valuable insight into the mechanisms of motilin receptor trafficking, it also offers an important generally applicable tool to investigate the tachyphylaxis-inducing properties of novel GPCR agonists at an early stage of drug discovery.

MATERIAL AND METHODS

Motilin receptor agonist compounds.

Human motilin was purchased from American Peptide Company, and erythromycin was supplied by Sigma. Synthesis of BMS-591348 has been described (compound 11a in Li et al., 2004). A research sample of ABT-229 was kindly provided by Abbott Laboratories.

Construction of the expression vector pMR-EGFP.

Human motilin receptor cDNA (MR; GeneBank accession # NM_001507) was amplified by PCR using the following primer set to introduce two restriction sites (*Eco*RI and *Bam*HI) at the 5' and 3' ends, respectively:
CCGGAATTCATGGGCAGCCCCTGGAAC;
CGCGGATCCCATCGTCTTCACGTTAGC. The PCR product was cloned into the TA cloning® vector pCR2.1 (Invitrogen), digested with *Eco*RI and *Bam*HI (Gibco BRL), and inserted into the expression vector pEGFP-N3 (Clontech) previously digested with the same restriction enzymes. The sequence was

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confirmed by DNA sequencing. Plasmid purification was performed using the Endotoxin free Maxi Preparation kit (Qiagen).

Cell culture.

Human embryonic kidney cells (HEK-293, American Tissue Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM, Mediatech Inc.) supplemented with 10% fetal bovine serum (Mediatech Inc.), 1x essential amino acids (Mediatech Inc.), and 1.0 mM sodium pyruvate (Mediatech Inc.). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

Stable expression of MR-GFP in HEK-293 cells.

$5x10^6$ cells were grown overnight onto poly-D-lysine coated 100 mm plates (BD Biosciences). Cells were transfected with 12.5 μg of purified plasmid DNA and 50 μl of lipofectamine 2000 in 1000 μl media as described in the manufacturer's instructions. 48 hrs after transfection, cells were split onto 3 plates, and 24 hrs later selection was applied via addition of 400 μg /ml geneticin (Life Technology, Inc). Transfected cells were maintained for 2 weeks in selection media containing 400 μg /ml geneticin. MR-GFP expressing cells were identified by epifluorescence microscopy and sorted by flow cytometry. Selected cells were pooled and expanded, yielding a polyclonal population containing less than 1% cells that were not expressing MR-GFP.

Motilin membrane binding assay.

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Membranes were prepared from HEK-293 cells expressing MR-GFP and HeLa-MR9 cells expressing wild-type MR (Li et al., 2004) by homogenization in 50 mM Tris-HCl buffer, pH 7.4 containing 10 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM PMSF and 1 mg/l aprotinin in a Polytron homogenizer at 13,000 rpm for 2 min followed by centrifugation at 740 X g for 15 min and centrifugation of the supernatant at 140,000 X g for 30 min. The membrane pellet was washed, recentrifuged and suspended in homogenization buffer. Binding of motilin to these membranes was assayed by homogeneous Scintillation Proximity assay (SPA). To each well of a 96-well OptiPlate (Packard), 50 µl of 2x assay buffer (50mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM PMSF, 5 mM KCl, 0.1% BSA, 2 mM CHAPS); 20 µl of cell membranes (2.5 µg); 15 µl of 10x [^{125}I]-motilin (Amersham Pharmacia, IM 337, specificity 2000 Ci/mmol, increasing concentrations); 15 µl of buffer or unlabeled motilin (1000X, for non specific binding); 0.5 mg wheat germ agglutinin coated polyvinyltoluene SPA beads (Amersham, RPNQ 0001) in 50 µl of 1x assay buffer were added. Contents in 150 µl of total volume were incubated at room temperature overnight. Plate was counted in TopCount for 1 min/well to determine the radioactive ligand bound to the receptor.

Internalization assay.

HEK-293 cells expressing MR-GFP were seeded at a density of 4x10^4 into black, clear view glass bottom poly-D lysine coated 96 well plates (Becton and Dickinson) and grown overnight. The cells were incubated at 37°C with synthetic human motilin (American Peptide Company) at the indicated concentrations in

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assay buffer (Hepes buffered DMEM (Mediatech Inc.), 1x essential amino acids, 1.0 mM sodium pyruvate, and 0.1%BSA). At the indicated time points cells were washed twice with ice cold PBS containing calcium and magnesium (Mediatech, Inc.), and then fixed with 4% paraformaldehyde for 15 min at room temperature. Fixation buffer contained 10 μg/ml Hoechst 33342 (Molecular Probes) to stain the nuclei. Finally, cells were washed twice with PBS, sealed, and stored at 4°C. Internalization of cell surface receptor was confirmed by confocal laser scanning microscopy (LSM 510, Zeiss) and quantified with a High Content Screening (HCS) Array Scan instrument (Cellomics, Inc) using a GPCR signaling algorithm (see "Quantification of Receptor Internalization"). Dose response was assessed by incubating the cells for 45 min at 37°C in 50 μl assay buffer containing increasing concentrations of motilin. For the internalization time course, cells were incubated at 37°C with 100 nM motilin for 5, 10, 20, 30, and 40 min. To label lysosomes, HEK-293 MR-GFP cells were incubated with 50 nM lysotracker (Molecular Probes) for the indicated time periods. To assess the effect of cytoskeletal inhibitors on MR-GFP internalization, HEK-293 MR-GFP cells were primed with 10 μM cytochalasin D or nocodazole (Sigma) for 60 min. The cells were then washed twice with ice cold PBS and further incubated with 10 or 100 nM motilin for 45 min in the absence of inhibitors.

Redistribution of internalized MR-GFP to the plasma membrane.

Cells were incubated 37°C with 10 or 100 nM motilin for 15 min. Internalization of MR-GFP was confirmed by confocal microscopy. Subsequently, cells were

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washed twice with ice cold PBS containing calcium and magnesium (Mediatech, Inc.) and placed in assay buffer for 15, 30, 90, 300 min prior to fixation.

Confocal laser scanning microscopy.

Images were acquired using a Zeiss Axiovert 200 LSM510 confocal microscope workstation equipped with a 63x (numerical aperture=1.4) objective and argon/krypton laser excitation source. GFP was visualized using a standard FITC 488 nm excitation and BP 505-550 nm emission filter. Texas red-conjugated lysotracker was visualized using a standard texas red 543 nm excitation filter and a LP650 nm emission filter. Hoechst DNA staining was visualized using a standard UV 364 nm excitation filter and a BP 385-470 nm emission filter. Confocal images (512x512x8bits) were acquired by averaging 8 scans per line in a series of confocal planes. Three-dimensional images were reconstructed using all of the acquired confocal planes.

Quantification of receptor internalization.

MR-GFP internalization as a function of ligand exposure was quantified using an HCS Array Scan (Cellomics, Inc.). Images were acquired using a Zeiss Axiovert 200 epifluorescent microscope with a 20 X objective embedded in the instrument. A GPCR signaling algorithm package for the Norak™ β-arrestin technology was adapted to measure the percentage of cells responding to motilin treatment in each well. The algorithm identifies individual cells (valid objects) based on their Hoechst-labeled nuclei and draws a nuclear mask around each nucleus. Nuclear

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mask identification is dependent on the nuclear area, length/width ratio, and average of total Hoechst fluorescence intensity. Cellular domains are defined by dilation of each nuclear mask with a user defined number of pixels, limited by the MR-GFP plasma membrane staining in unstimulated cells. Upon ligand stimulation, internalized MR-GFP vesicles appear as fluorescent spots. Valid MR-GFP Spots are identified by their area, length/width ratio, fluorescence intensity, and must reside within a cellular domain in order to be counted. To classify cells as responders, non-responders, or non-identifiable objects, each valid object is sequentially tested against a set of user defined thresholds. The total fluorescent spot area per cell has to exceeded a minimum value (as defined in un-stimulated control cells) in order to be classified as responder. Cells that fail this criteria are further analyzed based on their cellular texture. This threshold is determined by measuring the non-uniformity of MR-GFP distribution in unstimulated cells. Cells that do not qualify as non-responders are dropped from the selected object count, but still count as a valid objects (non-identifiable). The algorithm reports selected object count, valid object count, response phase classification, and percent responders. Percent responders are calculated by the total number of responder cells in a well divided by the selected object count. 99% of valid cells were selected and classified either as responders or non-responders. 500 cells were counted per well, and six replicate wells were measured for each treatment.

Quantification of Ca^{2+} Signaling (FLIPR-Assay).

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The stable cell line HeLa-MR9 expressing human motilin receptor was used to measure motilin-receptor induced Ca^{2+} signaling (Li et al., 2004). A detailed description of the fluorescence imaging plate reader (FLIPR) protocol and tachyphylaxis (receptor desensitization) experiments has been reported previously (Li et al., 2004).

RESULTS

Expression of motilin receptor GFP chimera (MR-GFP) in HEK-293 cells.

A GFP-motilin receptor chimera was generated in order to directly study the agonist-induced cellular trafficking pattern of the human MR. The cDNA for MR was fused N-terminally to an EGFP (MR-GFP) sequence in a mammalian expression vector under the control of the CMV promotor. Subsequent transfection of HEK-293 cells, followed by appropriate selection (see Material and Methods), yielded a stable polyclonal cell population expressing MR-GFP. When analyzed by confocal fluorescence microscopy, HEK-293 MR-GFP cells showed green fluorescence localized to the cell membrane with minimal intracellular accumulation in the absence of ligand (Fig. 1A). Furthermore, stimulation with 100 nM motilin for 45 minutes resulted in pronounced receptor internalization, manifested by the appearance of intracellular fluorescent spots (Fig. 1B). Control treatments of HEK-293 MR-GFP cells with other peptide ligands such as neurotensin did not induce receptor internalization (data not shown).

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To determine the affinity of the ligand to MR-GFP and compare it with wild-type MR, a saturation binding experiment with increasing concentrations of [^{125}I]-motilin was carried out using a scintillation proximity assay (SPA). Membrane fractions were harvested from HEK-293 MR-GFP and HeLa-MR9 cells, a stable cell line expressing wild-type motilin receptor (Li et al., 2004). The determined affinity constant (K_d) for wild-type MR and MR-GFP membranes was 4.4 nM, suggesting that motilin binding occurs with similar affinities for both receptors (Fig. 1C). In addition, the inhibitory constant (K_i) determined for motilin in competition binding assays was 11.0 nM and 9.9 nM for wild-type MR and MR-GFP, respectively (data not shown). Thus, the MR-GFP construct appears to be identical to wild-type MR with respect to motilin binding.

Internalization of the MR-GFP is dose-dependent.

Next, the dose dependence of MR-GFP internalization was determined. Cells expressing MR-GFP were treated with assay buffer alone, or buffer containing 2, 5, 10, 50, and 100 nM motilin for 45 min. A dose-dependent increase in intracellular vesicles labeled by MR-GFP was observed, together with a corresponding decrease in fluorescence at the cell surface (Fig. 2A). Maximal receptor internalization was stimulated by 100 nM motilin. Internalization was quantified by assessing the percentage of cells responding to motilin treatment using the Cellomics Array Scan instrument. Incubation with ascending concentrations of motilin (2.5, 5, 50, 80, 160, 320, and 640 nM) resulted in a gradual increase in the number of cells containing intracellular vesicles

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(responder cells; Fig. 2B). The Cellomics Array Scan data analysis showed that dose-response saturation occurred at 80 nM motilin, similar to observations using the laser scanning confocal microscope. The half-maximal effective motilin concentration (EC_{50}) for receptor internalization was 19 nM, comparing well to the Ca^{2+}-signaling EC_{50} of 9.2 nM reported for a similar MR-GFP chimera (Thielemans et al., 2005).

Time-dependent internalization of MR-GFP.

The time course of MR-GFP internalization was determined for a dose that stimulates the maximum response. Cells were incubated in 96-well plates with 100 nM motilin and fixed at 5 minute time increments. A time-dependent gradual increase in accumulation of intracellular vesicles was observed within 15 min of ligand exposure (Fig. 3A). Array Scan quantification confirmed that formation of MR-GFP vesicles occurs with motilin application, and showed that more cells responded as the exposure time increased (Fig. 3B). After 45 minutes, 95% of the cells internalized receptor in response to 100 nM motilin.

Internalized MR-GFP is redistributed to the plasma membrane.

To determine the fate of receptor after internalization, HEK-293 MR-GFP cells were incubated with 10 or 100nM motilin for 15 minutes at 37°C. At this time point internalization of MR-GFP into vesicles is initiated but not maximally stimulated (compare Fig. 3). Subsequently, MR-GFP internalization was stopped by washing out residual ligand. The cells were incubated in assay buffer at 37°C,

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and the endocytosed MR-GFP population was studied by confocal microscopy at different time points. Initial receptor internalization, stimulated with 10 nM motilin for 15 minutes, is shown in Fig. 4A (0 minute time point). A decrease in fluorescent vesicles occurred within 15 minutes after ligand withdrawal (Fig. 4A, 15 minute time point). These effects were dose dependent, as shown in Fig. 4B, where treatment with 100 nM motilin resulted in more pronounced receptor internalization. The number of internalized MR-GFP containing vesicles gradually decreased over 30 minutes after ligand withdrawal, and no vesicles were detectable after 90 minutes (Fig. 4B).

In order to determine whether internalized MR-GFP vesicles were targeted for lysosomal degradation, we performed subcellular colocalization experiments upon ligand exposure. HEK-293 MR-GFP cells were co-incubated with 100 nM motilin and lysotracker red for 45 min at 37°C prior to chemical fixing. Subsequently, cross-sections along the z-axis from the bottom to the top of the cells were imaged by confocal microscopy to visualize the appropriate fluorescent signals. Figure 5 shows a cross-section from the top third part of the cell, close to the mid-section (section 31 from a total of 45 slices). The ligand-induced MR-GFP spot pattern (Fig. 5A) was distinct from the lysosomal distribution (Fig. 5B), and the fluorescent signal for lysotracker red did not colocalize with MR-GFP in the respective overlay of both images (Fig. 5C). Furthermore, when the fluorescence intensities for both colors were plotted along a section through the cells (black arrow, Fig. 5C), the respective maximal intensities did not overlap, lending further evidence for a lack of MR-GFP vesicle

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co-localization with lysosomes. The same result was consistently observed in other cross-sections along the cellular z-axis (data not shown). These results suggests that endocytosed MR-GFP is not targeted for lysosomal degradation under conditions of prolonged exposure to excess ligand. Since internalized MR-GFP vesicles disappear with time after ligand withdrawal (Fig. 4) but fail to co-localize with the lysosomal compartment (Fig. 5) we conclude that MR-GFP molecules are recycled back to the plasma membrane after ligand-induced endocytosis.

Microtubules are essential for internalization of MR-GFP.

The role of the cytoskeletal network during internalization was investigated using the microtubules disrupting agent nocodazole and the microfilament inhibitor cytochalasin D. HEK-293 MR-GFP cells were pre-incubated with nocodazole or cytochalasin D (10 μM) for 60 min prior to treatment with 10 or 100nM motilin for 45 min. Pre-incubation with cytochalasin D had no effect on MR-GFP internalization (data not shown), indicating that microfilaments are not directly involved in MR-GFP endocytosis. However, nocodazole pretreatment for 60 minutes profoundly disturbed the MR-GFP internalization pattern. After depolymerization of microtubules and stimulation with 10 or 100nM motilin, MR-GFP containing vesicles accumulated close to the plasma membrane (Fig. 6B and D, respectively). These MR-GFP containing vesicles were larger compared to internalization controls without inhibitor (Fig. 6A, C), and may represent local clusters of ligand-bound MR-GFP molecules that are prevented from budding off

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the plasma membrane and being internalized. Similar effects of nocodazole on receptor internalization were reported for a parathyroid receptor-GFP chimera (Conway et al., 2001).

The redistribution time-course of internalized MR-GFP varies with different agonists.

Several compounds have been shown to mediate a pharmacological response through their interaction with the motilin receptor. These include the motilides Erythromycin-A and its derivative ABT-229, as well as a series of novel tetrahydrotriazolopyridazine-based amino acid derivatives (Li et al., 2004). An acute ligand exposure experiment was performed in order to directly visualize how these various agonists affect the redistribution of internalized MR-GFP receptors. Cells were treated for 15 minutes with an equipotent dose (1000-fold EC_{50}) of each compound to achieve maximal receptor stimulation (150 nM motilin; 400 μM Erythromycin A; 4.2 μM ABT-229; 47 nM BMS-591348; (Li et al., 2004)). Subsequently, excess ligand was removed, and cells were further incubated at 37°C for 0, 15, 30, 90, and 300 minutes before fixation and confocal microscopy analysis. As shown in Fig. 7, MR-GFP containing vesicles were visible for all compounds after 0 and 15 minutes of ligand withdrawal. At 30 minutes, fluorescent vesicles induced by Erythromycin-A and motilin had partially disappeared whereas they persisted in cells treated with ABT-229 and BMS-591348. This effect was even more pronounced 90 minutes after ligand withdrawal when all MR-GFP containing vesicles had disappeared in motilin and

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erythromycin-treated cells. Interestingly, MR-GFP containing vesicles were still apparent in ABT-229 treated cells after a 300 minute wash-out period, in marked contrast to cells treated with the other three compounds (Fig. 7). These results demonstrate that the tested agonists elicit different redistribution rates of internalized MR-GFP molecules when applied at equipotent doses for Ca^{2+}-signaling (EC_{50}), ranking in the following order from slowest to fastest redistribution: ABT-229 > BMS-591348 > motilin > erythromycin.

Different agonists induce various degrees of motilin receptor desensitization (tachyphylaxis) as a function of initial dose and recovery-time.

The phenomenon of receptor desensitization, or tachyphylaxis, varies widely amongst different GPCR's. Significant signaling potency may remain after repeated agonist stimulation; alternatively, reduced ligand potency may result due to receptor uncoupling or internalization (Woolf and Linderman, 2003). In order to further evaluate the effects of MR internalization on its signaling properties, different motilin agonists were tested in a functional assay that measures intracellular Ca^{2+} concentration using a fluorescence imaging plate reader (FLIPR; Li et al., 2004). A stable cell line expressing MR was initially treated for 5 minutes with multiples of the respective EC_{50} of motilin, erythromycin, ABT-229, or BMS-591348 (Li et al., 2004), followed by ligand washout and a variable recovery period. Subsequently, the maximum functional response was measured with a secondary stimulation using a high dose of the

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respective ligand (100-fold of the EC_{50}). As seen in Fig. 8A, increasing initial doses of motilin resulted in a stepwise reduction of peak Ca^{2+} responses upon secondary stimulation after a 30 minute recovery period. Interestingly, maximal MR signaling at secondary stimulation was dependent on the initial dose as well as on the recovery period duration: The highest initial dose of motilin, 100-fold EC_{50}, required the cells to recover for over 300 minutes in order to regain maximal signaling capacity, whereas 1x EC_{50} required only 30 minutes. Identical experiments performed with erythromycin, ABT-229, and BMS-591348 showed similar dose-dependency for the secondary response, insofar that high initial ligand doses reduced subsequent receptor signaling amplitude (Fig. 8B-D, respectively). Secondary signaling responses were also time-dependent for all compounds. Interestingly, the potent MR agonist ABT-229 showed only ~20% maximal signaling after 24 hours when stimulated at 100-fold EC_{50} (Fig. 8C). These desensitization effects did not correlate with potency since a more potent agonist, BMS-591348, showed ~40% of initial signaling after a 6 hour recovery period, and achieved maximal initial signaling by 24 hours (Fig. 8D). Erythromycin required the shortest recovery period; peak MR signaling was recorded after 30 minutes, regardless of initial dose (Fig. 8B).

Based on these results it can be concluded that MR desensitization is dose- and time-dependent. Each agonist showed a distinct receptor desensitization profile, ranking in the following order from strong to weak tachyphylaxis-inducing properties: ABT-229 > BMS-591348 > motilin > erythromycin.

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DISCUSSION

Regulation of seven-transmembrane receptor signaling and endocytosis has been studied extensively, revealing multiple mechanisms that contribute to the remarkable diversity of physiological responses mediated by small molecule or peptide ligands (von Zastrow, 2003). While a classical model of GPCR internalization has emerged, several receptor-specific variations thereof have been described (Pierce et al., 2002). Four major phases can be distinguished that most GPCRs undergo upon ligand-activation: acute signaling, rapid desensitization, endocytosis, and post-endocytic sorting (reviewed in von Zastrow, 2003; Marchese et al., 2003). First, binding of agonist promotes the release of heterotrimeric G-proteins from the receptor, initiating signaling through the activation of second-messenger pathways (e.g. ion channels, adenylyl cyclase, kinase cascades). Subsequently, receptors are phosphorylated by specific G-protein receptor kinases (GRKs) leading to the recruitment of β-arrestins, a family of GPCR signaling regulators (Pierce and Lefkowitz, 2001; Luttrell and Lefkowitz, 2002). This interaction prevents rebinding of G-proteins and attenuates receptor signaling. Arrestin-bound receptors associate with clathrin-coated pits in specific areas of the plasma membrane and are endocytosed. Finally, receptor vesicles undergo endocytic sorting and may be targeted to a recycling pathway mediating resensitization of receptors, or to a lysosomal degradative pathway mediating receptor downregulation.

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Post-endocytic sorting information for GPCRs is contained within the cytoplasmic C-terminal tail. For example, the protease-activated receptor-1 (PAR1), normally sorted to lysosomes, was routed to the plasma membrane when fused to the cytoplasmic tail of the recycling substance P receptor (Trejo and Coughlin, 1999). Furthermore, it appears that the interaction of β-arrestin with the receptor determines in part the kinetics of GPCR recycling (Marchese et al., 2003). GPCRs can be classified based on whether arrestins travel into endocytic vesicles along with receptors (class B GPCRs), or whether arrestins are confined to the periphery of the cells and do not colocalize with internalized receptors (class A GPCRs). Classical examples of class A receptors are the β2-adrenergic, dopamine D_{1a}, endothelin and μ-opioid receptors (Zhang et al., 1999; Oakley et al., 2000). In contrast, the angiotensin, substance-P, neurotensin, thyrotropin-releasing hormone (TRH), and vasopressin-V2 receptors belong to class B and contain stretches of serine and threonine residues that are proposed to mediate high-affinity arresting binding upon phosphorylation (Oakley et al., 2001). Prolonged association of arrestin with receptors might delay GPCR dephosphorylation, and thereby influence the kinetics of receptor resensitization and recycling. Both the angiotensin and vasopressin-V2 receptors recycle very slowly (hours) compared to class A receptors (minutes) (Oakley et al., 1999; Anborgh et al., 2000). The amino acid sequence of the motilin receptor C-terminal tail does not appear to contain distinct S/T clusters as described by Oakley et al. (2001). Furthermore, co-immunoprecipitation experiments using anti-GFP antibodies failed to detect β-arrestin in MR-GFP cells that were

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stimulated with ligand (data not shown). The combination of these observations suggest that MR belongs to the class A recycling receptor family, or may internalize via a β-arrestin independent mechanism (Pierce et al., 2002).

When MR-GFP endocytosis was induced by motilin administration, the internalized fluorescent vesicles disappeared rapidly upon ligand withdrawal (see Fig. 4). Co-localization experiments failed to detect redistribution of MR-GFP vesicles to lysosomes under conditions of chronic motilin exposure (see Fig. 5). Since internalized MR-GFP vesicles disappear over time but are not targeted for degradation via the lysosomal pathway we conclude that MR-GFP is recycled back to the plasma membrane. Although this is an indirect conclusion it is consistent with all our observations, including MR-GFP labeling of the plasma membrane after the disappearance of intracellular fluorescent vesicles (Fig. 4, time points 90 and 300 min). However, it is important to consider the large overexpression of MR-GFP in this context. This is exemplified by residual fluorescence staining of the plasma membrane even under conditions of prolonged exposure to excess ligand (see Fig. 1, 2, 3 at 100 nM motilin). This implies the existence of a separate MR-GFP pool that is unable to be internalized, most likely because accessory molecules involved in GPCR endocytosis become limiting under conditions of excess ligand and maximal rate of MR-GFP internalization (e. g. β-arrestin, GRKs, or others). Hence, receptor recycling cannot be inferred purely from the occurrence of MR-GFP in the plasma membrane after ligand withdrawal, since this could be explained by a residual receptor population that never entered the cytoplasm. Attempts to label

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MR-GFP with biotin derivatives at the cell surface failed (data not shown), which may be explained by the complete lack of lysine residues in the extracellular domains of MR. In the absence of further methods to directly corroborate receptor recycling, our experiments provide good evidence that internalized MR-GFP molecule are routed back to the plasma membrane.

A wide variety of GPCR assays have been developed to screen compound libraries against known and orphan receptors, and most common cell-based assay formats utilize the GPCR signal transduction pathways to generate a readout of receptor signaling (reviewed in Cacace et al., 2003; Kenakin, 2003; Chalmers and Behan, 2002; Robas et al., 2003). These assays are robust and allow the identification of potent and selective ligands, but do not offer critical information on receptor trafficking. More recently, the advent of high content biology has opened up the possibilities for the development of GPCR assays based on single cell imaging (Milligan, 2003). Generating chimeras between a polypeptide of interest and green fluorescent protein (GFP) enables automated image analysis. The increased throughput of these machines, compared to classical confocal microscopy, allows the quantitative analysis of specific biological processes. Furthermore, assays can be multiplexed by using fluorescent dyes or proteins with distinct emission properties (Abraham et al., 2004). In this study an internalization assay was developed by tagging motilin receptor with a C-terminal GFP moiety. This strategy did not appear to substantially alter MR sensitivity to ligand (Fig. 1C) or internalization after stimulation (Thielemans et al., 2005), similar to reports for other GPCRs

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(Milligan, 1999; Kallal and Benovic 2000; Kallal et al., 1998). Its primary advantage is that it enables automated analysis of receptor endocytosis directly, as opposed to indirect methods that follow GPCR trafficking via the redistribution of β-arrestin-GFP chimeras (Oakley et al., 2002). Furthermore, it is relevant that the assay performed reproducibly on different cell populations, as illustrated by the small variation within 6 replicates of 500 cells counted per well (Fig. 2B, 3B). Finally, the assay is amenable to 96- or 384-well format, achieving respectable throughput sufficient for a secondary screen for use during the lead optimization phase of the drug discovery process.

Motilin receptor represents an attractive drug target for the treatment of functional gastrointestinal disorders (Chovet, 2000; Sanger and Hicks, 2002; Camilleri, 2002; Maganti et al., 2003). However, the clinical utility of chronic MR agonist administration has been limited by the lack of efficacy after prolonged exposure, as demonstrated by ABT-229 (Talley et al., 2000). It is possible that MR tachyphylaxis was the primary cause for lack of efficacy, although gastroparesis clinical trials have been criticized for their small sample sizes, uncontrolled designs, short duration, and inadequate symptom assessment (Maganti et al., 2003; Tack and Peters, 2001; Camilleri, 2002). Clinical data show that a single dose of ABT-229 strongly increased gastric emptying after the first meal in healthy volunteers, but no effect was observed after the second meal despite the presence of considerable residual drug concentrations in the serum (Verhagen et al., 1997). Such clinical evidence of functional motilin receptor desensitization has been reproduced in cell cultures using ABT-229 and other

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agonists (Li et al., 2004). More recently, by conducting a series of elegant structure-activity relationship experiments, Thielemans *et al.* (2005) were able to identify a specific hydroxyl group within the ABT-229 molecule that appears to mediate the majority of its MR desensitizing properties. Furthermore, the authors demonstrated that agonist potency alone is not the sole determinant for its ability to desensitize, internalize, and re-sensitize the motilin receptor (Thielemans et al., 2005). Our results presented here are consistent with these findings, demonstrating that ligand potency and tachyphylaxis properties are not necessarily linked; instead, we find a correlation between prolonged receptor internalization, delayed redistribution to the plasma membrane and tachyphylaxis. SInce EC_{50} values are a composite of ligand affinity and efficacy, it may be important to deconvolute these properties by measuring true receptor affinity and occupancy. MR internalization and intracellular trafficking are influenced by ligand on- and off-rates, both at the cell surface as well as in endocytic vesicles. Radiolabeling of compounds may generate tools to further investigate this complex relationship.

In conclusion, our *in vitro* results provide evidence that intracellular trafficking of identical motilin receptor molecules varies substantially with different ligands according to their distinct agonist properties. Importantly, considering the clinical importance of tachyphylaxis for this specific drug target, our findings support accumulating evidence that it is possible to discover potent motilin receptor agonist leads with reduced receptor desensitization properties *in vitro* compared to ABT-229. Finally, the results emphasize the utility of a high content

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internalization assay as an appropriate secondary screen in order to facilitate the development of MR agonists with sustained efficacy, which will hopefully minimize clinical trial failure in the near future.

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Figure Legends

Fig 1: Characterization of MR-GFP chimera stably expressed in HEK-293 cells. Confocal microscopy pictures of stable HEK-293 MR-GFP cells either treated with assay buffer alone (A) or with 100 nM motilin (B) for 45 min at 37°C. (C) Saturation binding of [^{125}I]-motilin to HEK-293 MR-GFP membranes (2.5 μg; circles) and Hela-MR9 membranes (2.5 μg; inverted triangles) containing wild-type motilin receptor. Binding was determined in a scintillation proximity assay (SPA) by subtracting nonspecific binding in the presence of 1000-fold excess unlabeled motilin from the total binding, and plotted against the radioligand concentration. Valueas are mean +/- SD of four experiments in duplicate. The curves were fitted by hyperbola equation in SigmaPlot.

Fig 2: Internalization of MR-GFP in response to increasing concentrations of motilin. Stable HEK-293 MR-GFP cells were treated in 96-well plates with increasing concentrations of motilin as indicated for 45 min at 37°C. After fixation cells were visualized by confocal microscopy (A) and subsequently analyzed with the Cellomics Array Scanner to determine the percentage of cells containing internalized MR-GFP molecules (motilin responders; see Materials and Methods). A dose-response curve plotting percentage of responder cells as a function of motilin concentration is shown in (B). Each data point represents mean +/- SD of six replicated wells, counting 500 valid cells per well.

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Fig 3: Time-dependent internalization of MR-GFP. HEK-293 MR-GFP cells grown overnight in 96-well plates were treated with 100 nM motilin for the indicated time periods at 37°C, fixed, and visualized by confocal microscopy (A). Subsequently, cells were subjected to Array Scan analysis and scored for MR-GFP endocytosis (see Material and Methods). MR-GFP internalization stimulated by 100 nM motilin is plotted as a function of incubation time (B). Each data point represents mean +/- SD of six replicated wells, counting 500 valid cells per well.

Fig 4: Redistribution of internalized MR-GFP upon acute motilin exposure. HEK-293 MR-GFP cells were treated with 10 nM (A) or 100 nM (B) motilin for 15 min at 37°C. Subsequently, cells were washed twice to remove excess ligand and further incubated in assay buffer for the indicated time periods before fixation and confocal microscopy analysis. Internalized MR-GFP vesicles disappeared over time and must have either been redistributed to the plasma membrane or degraded in the lysosomal compartment.

Fig 5: Internalized MR-GFP molecules do not overlap with lysosomal compartments upon motilin exposure. HEK-293 MR-GFP cells were co-incubated with 100 nM motilin and 1 ug/ml lysotracker red for 45 min at 37°C prior to fixation. Shown is a fluorescence microscopy cross-section along the z-axis of the cells (section 30 of a total of 45 sections of 0.1 μm thickness each, from the bottom to the top of the cells). A) MR-GFP staining (green), B) lysotracker red staining (red), C) overlay image of A and B. The maxima of the

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respective fluorescence intensities are plotted along a cross-section of the cells in C (black arrow), demonstrating minimal overlap of MR-GFP with lysosomal compartments.

Fig 6: Inhibition of MR-GFP internalization by nocodazole. HEK-293 MR-GFP cells were incubated for 60 min at 37°C either with vehicle (A, C) or with 10 μM nocodazole (B, D). Subsequently, cells were treated with 10 nM (A, B) or 100 nM (C, D) motilin for 45 min at 37°C, followed by fixation and confocal microscopy analysis.

Fig 7: Agonist-dependent variability of intracellular MR-GFP trafficking. Maximal internalization of MR-GFP was initiated by incubating HEK-293 MR-GFP cells with excess doses of motilin, erythromycin, ABT-229, or BMS-591348 for 15 min at 37°C (see text for details). After removal of unbound ligand cells were incubated at 37°C for 0, 15, 30, 90, or 300 minutes before fixation and confocal microscopy analysis. Note the agonist-dependent differences in receptor redistribution over time.

Fig 8. **Agonist-dependent variability of motilin receptor desensitization in FLIPR assays.** Primary stimulation (desensitization): HeLa-MR9 cells stably expressing the human motilin receptor were treated for 5 minutes with multiples of the corresponding EC_{50} (1x, 2x, 5x, 10x, 50x, and 100x) for either motilin (A), erythromycin (B), ABT-229 (C), or BMS-591348 (D). Secondary stimulation: After

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subsequent ligand washout and a variable recovery period of 30, 90, 300, or 1440 min cells were stimulated with a second addition of the respective compound at maximum effective dose (100-fold EC_{50}). The secondary Ca^{2+} signal was measured in FLIPR experiments and plotted as a percentage of initial response. Bars are ordered according to increasing initial dose from left to right for each recovery interval (1x EC_{50}, blue; 2x EC_{50}, red; 5x EC_{50}, yellow; 10x EC_{50}, green; 50x EC_{50}, dark red; 100x EC_{50}, orange). Receptor desensitization was dependent on initial ligand dose and recovery time, and differed substantially for each agonist.

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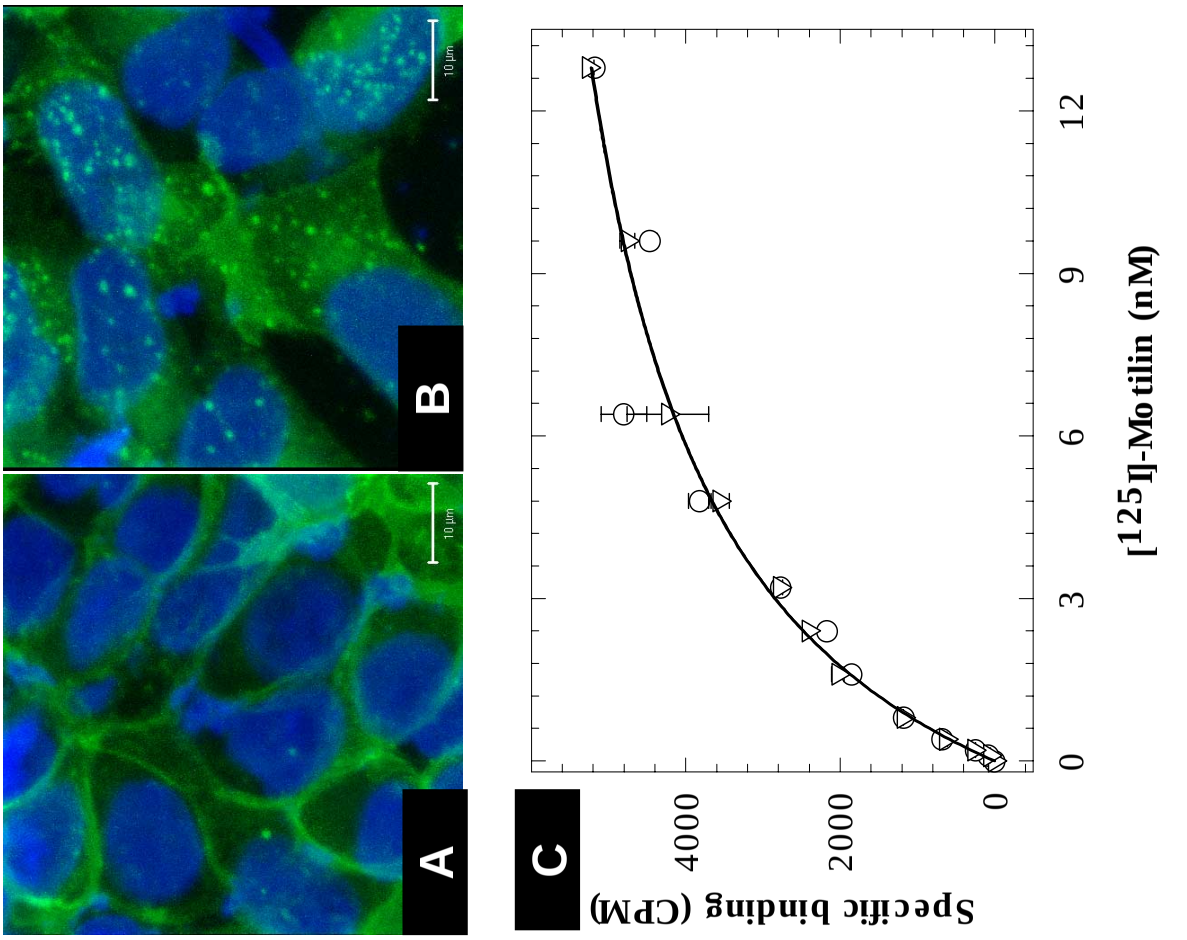
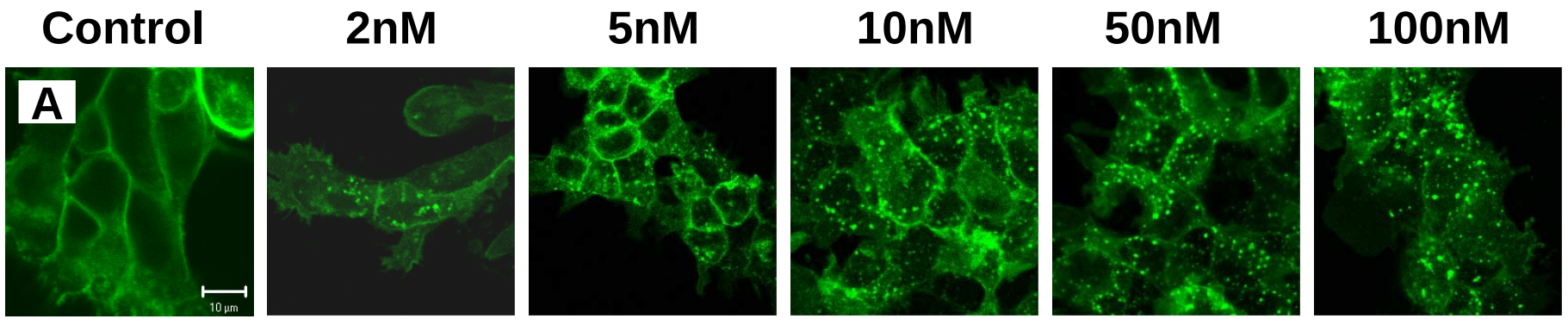
Figure 1





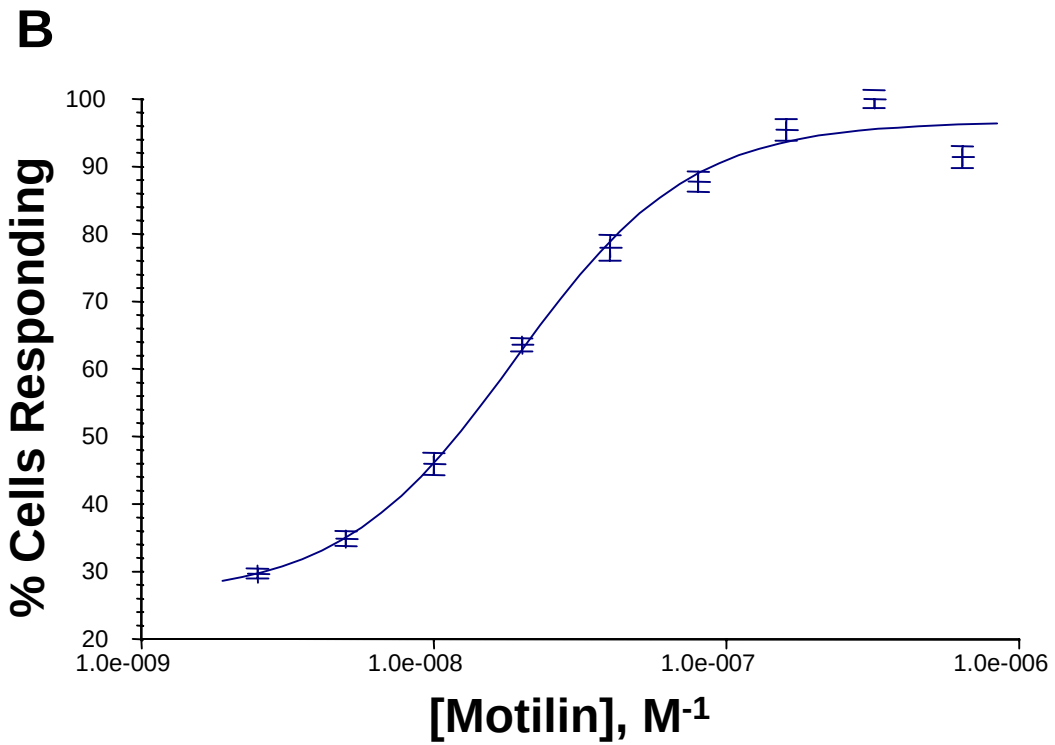


Figure 2

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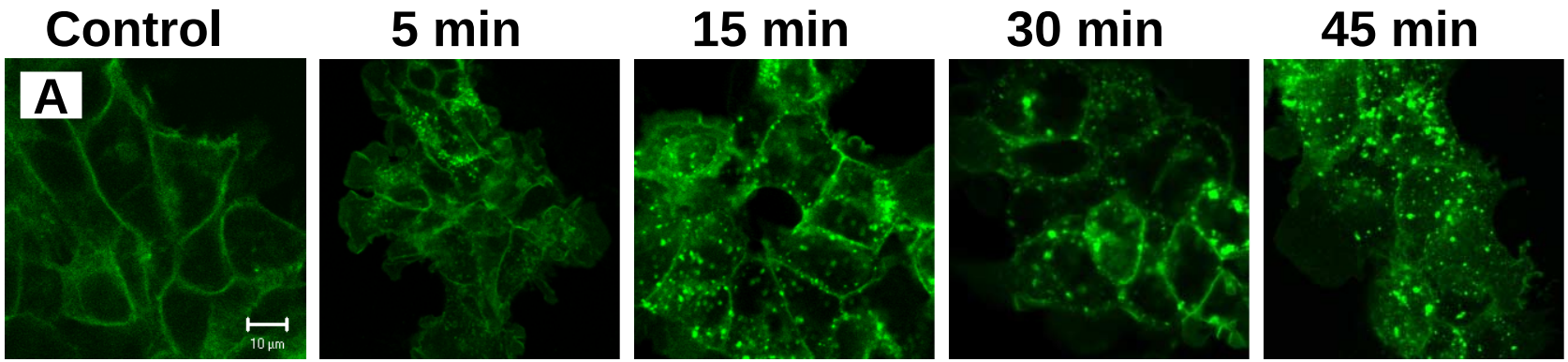

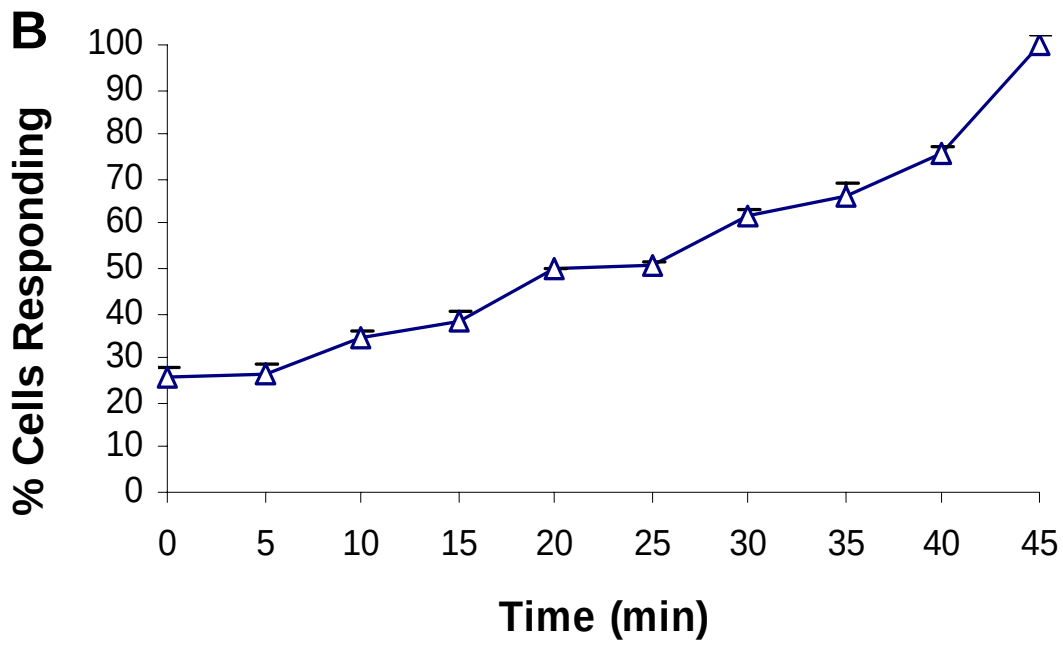

Figure 3

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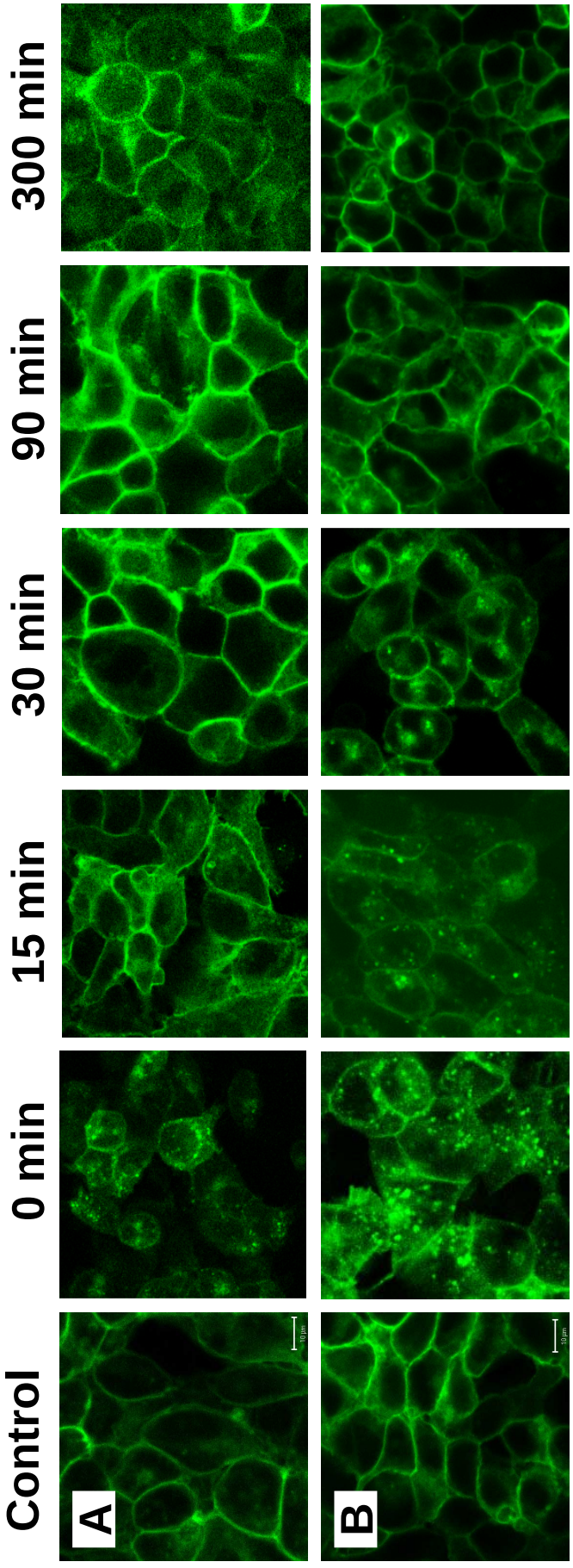

Figure 4

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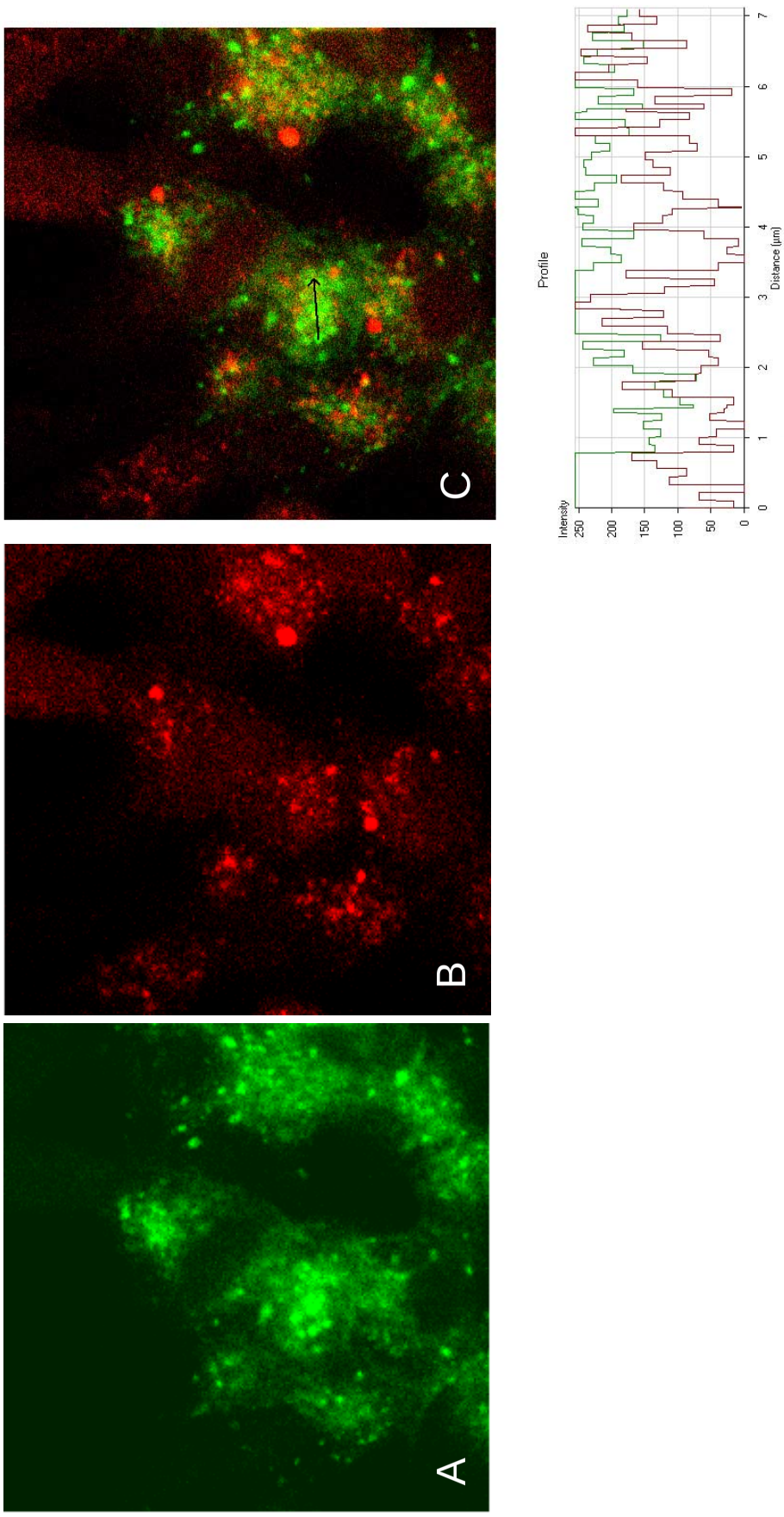

Figure 5

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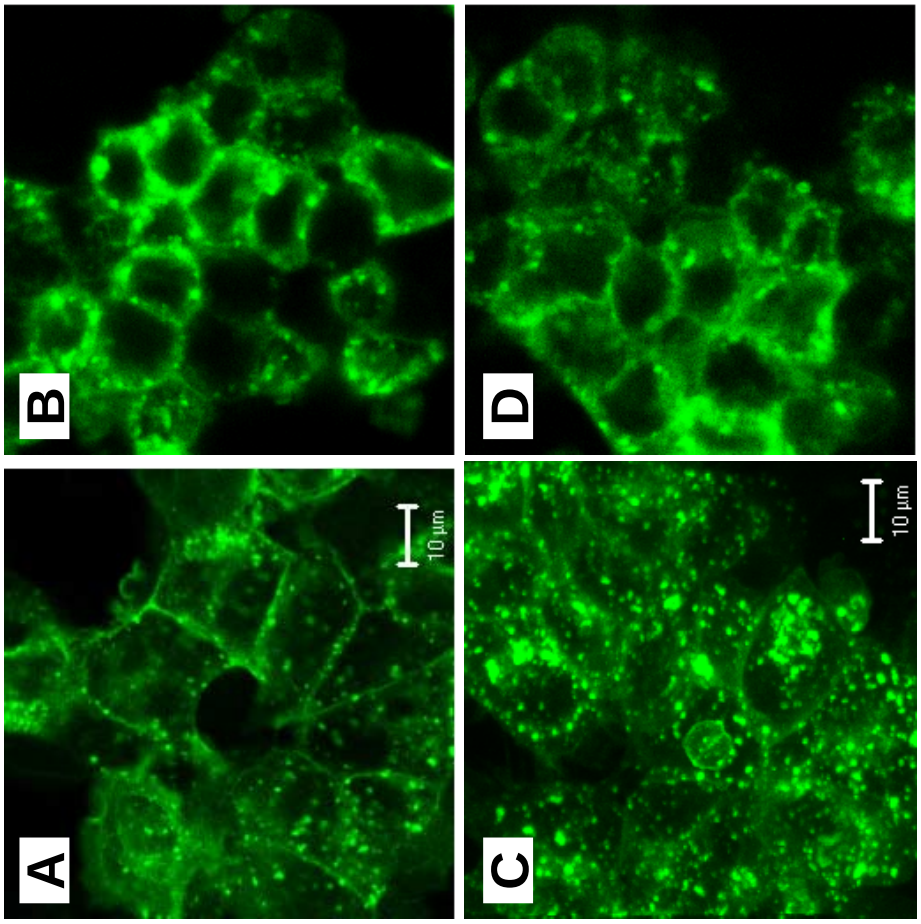
Figure 6



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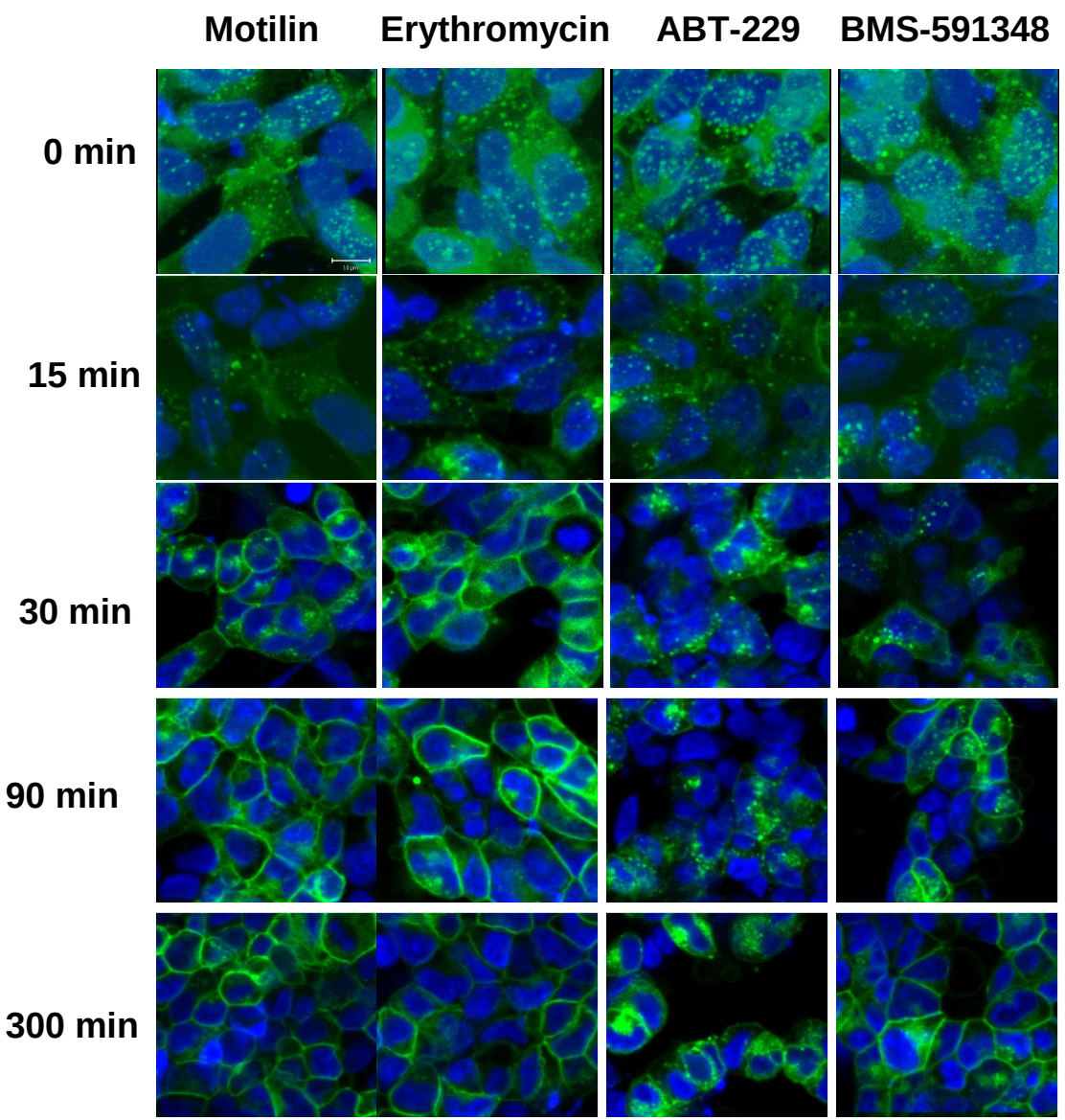

Figure 7

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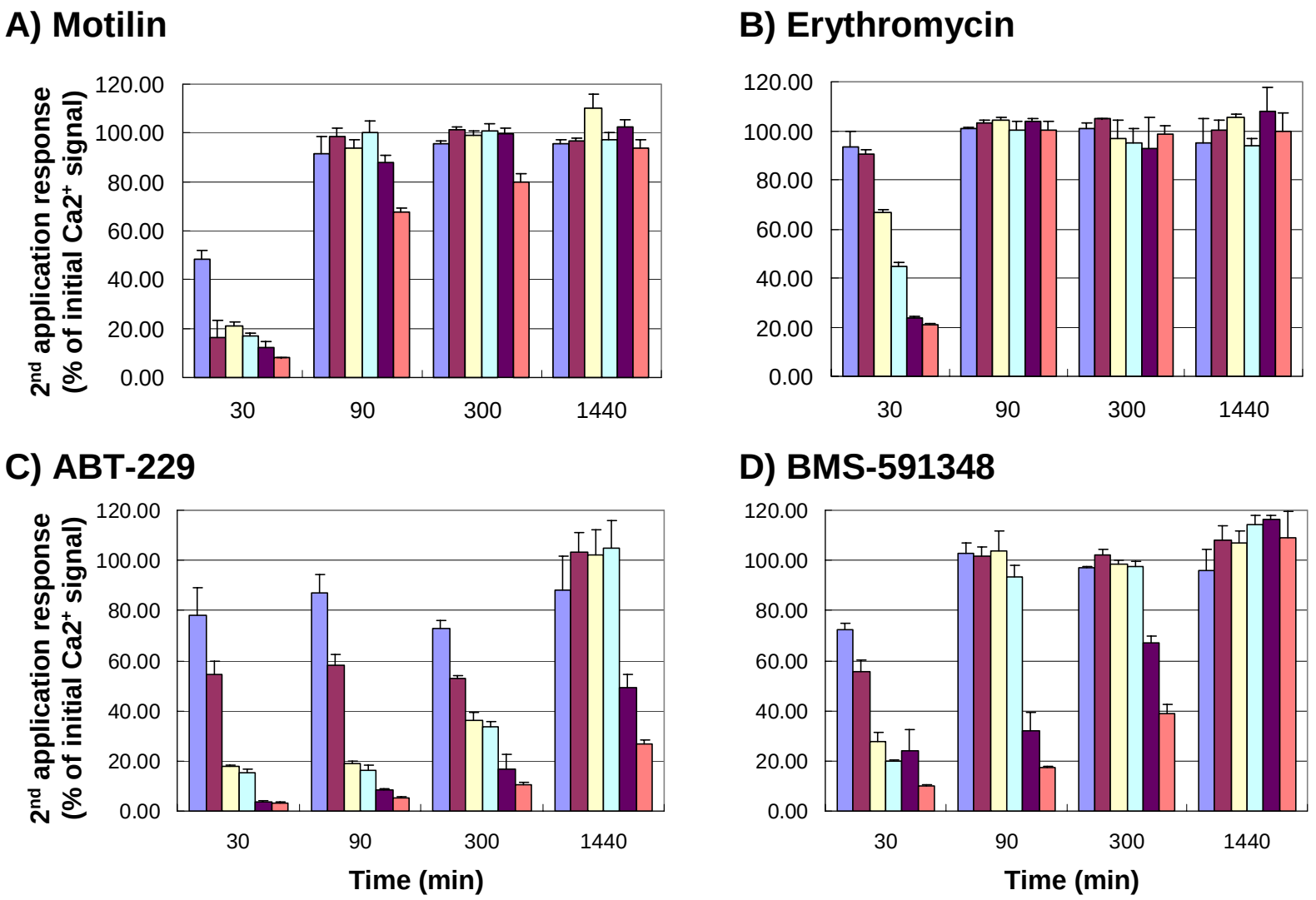

Figure 8

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