Role of sialic acids in feline enteric coronavirus infections


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To initiate infections, many coronaviruses use sialic acids, either as receptor determinants or as attachment factors helping the virus find its receptor underneath the heavily glycosylated mucus layer. In the present study, the role of sialic acids in serotype I feline enteric coronavirus (FECV) infections was studied in feline intestinal epithelial cell cultures. Treatment of cells with neuraminidase (NA) enhanced infection efficiency, showing that terminal sialic acid residues on the cell surface were not receptor determinants and even hampered efficient virus–receptor engagement. Knowing that NA treatment of coronaviruses can unmask viral sialic acid binding activity, replication of untreated and NA-treated viruses was compared, showing that NA treatment of the virus enhanced infectivity in untreated cells, but was detrimental in NA-treated cells. By using sialylated compounds as competitive inhibitors, it was demonstrated that sialylactose (2,6-α-linked over 2,3-α-linked) notably reduced infectivity of NA-treated viruses, whereas bovine submaxillary mucin inhibited both treated and untreated viruses. In desialylated cells, however, viruses were less prone to competitive inhibition with sialylated compounds. In conclusion, this study demonstrated that FECV had a sialic acid binding capacity, which was partially masked by virus-associated sialic acids, and that attachment to sialylated compounds could facilitate enterocyte infections. However, sialic acid binding was not a prerequisite for the initiation of infection and virus–receptor engagement was even more efficient after desialylation of cells, indicating that FECV requires sialidases for efficient enterocyte infections.

INTRODUCTION

Feline enteric coronavirus (FECV) is an enzootic enteropathogen in cats. The enteritis caused by its replication in intestinal epithelial cells is mild and mostly unnoticed (Addie & Jarrett, 1992; Pedersen et al., 1981). However, mutations in the viral genome can allow the virus to replicate efficiently in monocytes/macrophages, resulting in the fatal feline infectious peritonitis (FIP) (Dewerchin et al., 2005; Pedersen, 2009; Rottier et al., 2005; Stoddart & Scott, 1989; Vennema et al., 1998). Despite many attempts, treatment of FIP has remained palliative to date. In multi-cat environments, cat owners lose up to 10% of their cats, and recurrent FIP deaths are still a major reason to stop breeding programmes. As FIP is the consequence of mutations arising in the viral genome during a common FECV infection, FECV is an attractive target in the fight against FIP. Despite the valuable information available from different in vivo studies (Meli et al., 2004; Pedersen et al., 1981, 2008; Poland et al., 1996; Vogel et al., 2010), very little is known about the FECV–enterocyte interactions as these viruses had been uncultivable for many years. The propagation of these viruses in recently established feline intestinal epithelial cell cultures allows further unravelling of these FECV–enterocyte interactions in vitro (Desmarets et al., 2013).

Coronaviruses mediate their entry into host cells by their spike (S) proteins. Coronavirus S proteins have been shown to possess at least two receptor-binding domains (RBDs): the S1 N-terminal RBD and the S1 C-terminal RBD. Whereas the C-terminal RBD of most coronaviruses is involved in protein binding, the N-terminal RBD can act as a lectin, recognizing various sialic acids (Peng et al., 2011). Sialic acid binding has been described for members of the alpha-, beta-, and gammacoronaviruses. However, whereas some of these viruses rely on sialic acid binding for the initiation of host cell infections, others use sialic acids as attachment factors, but rely solely on another protein receptor to initiate their infections (Schwegmann-Wessels & Herrler, 2006).

Among alphacoronaviruses, a sialic acid binding capacity has been described for transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV). For both viruses, this sialic acid binding activity becomes more pronounced when virions are pretreated with neuraminidase (NA), demonstrating that the sialic acid binding site is masked by virion-associated sialic acids (Park et al., 2006).
The role of sialic acid binding during TGEV infections has been studied extensively. Sialic acid binding by TGEV Purdue is not essential in the initiation of in vitro infections as desialylation of cells hardly affects its replication, and mutants lacking the sialic acid binding site replicate to the same extent in cell cultures (Krempl et al., 1997; Schultze et al., 1996). However, when the absorption time is reduced, sialic acid binding contributes to efficient infection, showing that the sialic acid binding activity helps TGEV infections under unfavourable conditions, as encountered during its passage through the intestinal tract (Schwegmann-Wessels et al., 2011). Indeed, mutants lacking the sialic acid binding site were no longer capable of inducing enteropathy, showing that sialic acid binding is required to induce efficient intestinal infections in vivo (Bernard & Laude, 1995; Krempl et al., 1997), possibly by allowing the virus to interact with and pass through the mucus layer covering the epithelial cells (Schwegmann-Wessels et al., 2002). In contrast to TGEV Purdue, NA treatment renders cells more resistant to infection with TGEV Miller, bovine coronavirus (BCoV) (a betacoronavirus) and avian infectious bronchitis virus (IBV) (a gammacoronavirus), showing that these viruses use sialic acids as receptor determinants to initiate infection into host cells (Schultz & Herrler, 1992; Schwegmann-Wessels et al., 2011; Winter et al., 2006).

The role of sialic acids in feline coronavirus (FCoV) infections is unknown. However, it has been shown that FIP cats have hypsialylated serum α1-acid glycoprotein (AGP), whereas healthy cats in the same environment tend to have hypsialylated AGP, suggesting that sialylated compounds can determine the outcome of a FCoV infection, potentially by acting as a decoy for virus infections (Ceciliani et al., 2004; Paltrinieri et al., 2008). In the present study, the sialic acid binding capacity of FECV and the role of sialic acids in FECV infections was investigated in two different intestinal epithelial cell cultures with two different serotype I FECV strains.

RESULTS

Effect of NA treatment of cells on FECV infection

To assess the role of sialic acids as receptor determinants, cells were pretreated with 50 mU NA ml⁻¹ prior to inoculation with FECV strains UCD or UG-FH8. Surprisingly, removal of sialic acids greatly enhanced infectivity of both strains, even after 5 min absorption time (Fig. 1). This implies that FECV does not depend on terminal sialic acid residues on the enterocyte surface for the initiation of its infection and requires sialidases to allow efficient virus–receptor engagement.

When sialic acids are removed by NA treatment of cells, subterminal sugar residues are exposed. To investigate if these sugars were involved in the enhanced infection efficiency of FECV in desialylated cells, subterminal sugar residues, including D-galactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, were used as competitive inhibitors. None of these sugars (at concentrations up to 50 mM) reduced FECV infection in NA-treated cells, showing that attachment to one of these exposed sugars was not the reason for the enhanced infectivity of FECV (data not shown).

Effect of NA treatment of FECV on the replication in NA-treated and untreated cells

Knowing that NA treatment of coronaviruses can unmask sialic acid binding activity, the replication of untreated and NA-pretreated viruses was compared in both untreated and NA-treated cells. The effect of these treatments was analysed 12 h post-inoculation (p.i.) in ileocyte and colonocyte cultures for both FECV strains (Fig. 2). NA pretreatment of cells significantly enhanced infection efficiency of untreated viruses from 0.05 ± 0.05 to 3.63 ± 1.21% for UCD and from 0.59 ± 0.14 to 19.07 ± 18.86% for UG-FH8 in ileocytes. For the colonocytes, NA treatment of the cells increased the percentage of infected cells from 0.29 ± 0.29 to 3.36 ± 2.11% for UCD, and from 0.80 ± 0.19 to 33.45 ± 20.57% for UG-FH8. Although both strains were inoculated at comparable m.o.i. (0.25 and 0.35 as determined on NA-treated cells for UCD and UG-FH8, respectively), consistently more cells (ranging from 2.8 to 11.8 times more) were infected 12 h p.i. by UG-FH8 compared with UCD in both untreated and treated cells.

Removal of sialic acids from the virus enhanced infectivity for both strains in both untreated cell cultures, although not significantly for UCD in colonocytes. For ileocytes, treatment of the virus increased the number of infected cells on average seven times for UCD and 3.3 times for UG-FH8, whereas for colonocytes the percentage of infected cells was 1.6 times and 1.9 times higher for UCD and UG-FH8, respectively.
Desialylation of the cells also enhanced infection efficiency of NA-treated viruses, although not significantly for UG-FH8 in colonocytes. In contrast to untreated cells, NA treatment of the virus seemed to have a detrimental effect in NA-treated cells, although this reduction was only significant for UG-FH8 in colonocytes. Considering all treatments, inoculation of NA-treated cells with untreated virus resulted in the most efficient infection.

Effect of sialylated compounds on the replication of FECV in enterocytes

To further analyse FECV–sialic acid binding, and to investigate if the differences seen between untreated and NA-treated virions were due to sialic acid binding, the potential of different sialylated compounds [2,3-α-linked 2,6-α-sialyllactose, fetuin, porcine gastric mucin (PGM), bovine submaxillary mucin (BSM), and lactoferrin] to act as competitive inhibitors for FECV infection was studied. Therefore, both untreated and NA-treated virions were pre-incubated for 30 min with different concentrations of each compound before inoculation of colonocytes. Of all tested compounds, only sialyllactoses and BSM acted as inhibitors for FECV infection, whereas fetuin, lactoferrin and PGM had no effect on FECV infectivity at concentrations up to 200 μg ml⁻¹, 1 mg ml⁻¹ and 50 mg ml⁻¹, respectively.

![Fig. 2. Effect of NA treatment of FECV on the replication in untreated and NA-treated cells. For both untreated and NA-treated FECV, percentage of infection was evaluated 12 h p.i. in both untreated and desialylated cells. Data are expressed as the mean ± SD of the results of four separate experiments.](http://vir.sgmjournals.org)

Fig. 3 shows the relative percentage of infected cells 12 h p.i. after pre-incubation of both untreated and NA-treated viruses with different concentrations of 2,3-α-sialyllactose, 2,6-α-sialyllactose or lactose. In contrast to lactose, sialyllactoses significantly reduced infection of NA-treated viruses. For both strains, 2,6-α-linked sialic acids had a slightly more pronounced inhibitory activity compared with 2,3-α-linked sialic acids and reduced the absolute percentage of infected cells to a similar level as the untreated viruses. In contrast to the NA-treated viruses, untreated viruses were hardly affected by the sialyllactoses. Only for the UG-FH8 strain did 2,6-α-sialyllactose at the highest concentration (1000 μM) significantly reduce infection efficiency of the untreated virus. In desialylated cells, sialyllactoses did not have any effect on the replication of FECV (data not shown).

Whereas sialyllactoses notably inhibited infection of NA-treated virions, BSM was a very potent inhibitor of both NA-treated and untreated viruses, with almost complete inhibition of infection in untreated cells at a concentration of 1 mg ml⁻¹ (Fig. 4). Pre-incubation of only the cells with 2 mg BSM ml⁻¹ had no effect on viral replication, showing that BSM specifically interacted with the virus (data not shown). In addition, BSM also had an inhibiting effect on desialylated cells, although to a lesser extent than in untreated cells, indicating that it is more difficult for sialylated compounds to compete with the viral attachment.
The concentration needed to inhibit FECV infections in desialylated cells completely could not be determined, as toxicity was seen with BSM from 5 mg ml\(^{-1}\) onwards. Taken together, these results showed that FECV had a sialic acid binding capacity that was partially masked by virus-associated sialic acids and preferably recognized 2,6-\(\alpha\)-linked sialic acids over 2,3-\(\alpha\)-linked sialic acids. In addition, attachment to sialylated compounds could facilitate FECV infection, chiefly in the untreated enterocyte cultures, which can explain the increased infectivity of NA-treated viruses. However, it seemed that the receptor was more efficiently reached when sialic acid binding was reduced/avoided by desialylation of the cells, indicating that attachment to terminal sialic acid residues was not a prerequisite for the initiation of infection in vitro.

### DISCUSSION

Coronaviruses are able to attach to host cells in three different ways: viral lectin–host carbohydrate (e.g. spike–sialic acids), protein–protein (e.g. spike–aminopeptidase N (APN)), and viral carbohydrate–host lectin (e.g. mannose–DC-SIGN) interactions. In the present study, the sialic acid binding and the role of sialic acids in FECV infections of enterocytes was investigated. Sialic acid binding by the coronavirus S protein has been described for members of the alpha-, beta- and gammacoronaviruses, including TGEV, PEDV, BCoV, human coronavirus (HCoV) OC43, and IBV (Künkel & Herrler, 1993; Park et al., 2010; Schultze et al., 1991, 1992, 1996). In contrast to the alphacoronavirus TGEV, sialic acid binding is essential for initiating infection of host cells for members of both the beta- (BCoV and HCoV OC43) and gammacoronaviruses (IBV) (Schultze & Herrler, 1992; Schultze et al., 1996; Winter et al., 2006). For TGEV Purdue, it has been shown that NA treatment of the cells enhances APN binding, but this seems to have no enhancing effect on the viral infectivity in vitro (Schwegmann-Wessels et al., 2002; Shahwan et al., 2013). In addition, when absorption time is reduced, desialylation of cells even reduces infectivity, clearly showing the role of sialic acid binding under unfavourable conditions as encountered in the intestinal tract (Schwegmann-Wessels et al., 2011). In the present study, it was demonstrated that removal of sialic acids from cells greatly enhanced FECV infections in the enterocyte cultures, even after only 5 min absorption time. This shows that terminal sialic acid residues are not receptor determinants and that FECV interacts more efficiently with its (still unknown) receptor after desialylation of the cells. These results are valuable for the future propagation, titration and study of FECVs in cell cultures.

**Fig. 3.** Effect of sialyllactoses on the infectivity of untreated and NA-treated viruses in untreated colonocytes. Both untreated and NA-treated virions were pre-incubated for 30 min at 37 °C with 2,3-\(\alpha\)-sialyllactose, 2,6-\(\alpha\)-sialyllactose or lactose before inoculation. At 1 h p.i., the inoculum was removed by three washings and the relative percentage of infected cells was assessed 12 h p.i. Data are expressed as the mean ± SD of the results of four separate experiments. Significant differences (\(P \leq 0.05\)) are indicated with an asterisk.
Two different serotype I FECV strains were used in this study. It was noticed that UG-FH8 was more virulent compared with UCD in the enterocyte cultures. In comparison with UCD, UG-FH8 seemed to replicate and/or spread much faster, resulting in up to 31 times more infected cells 12 h p.i., although inoculated at comparable m.o.i. The impact of this virulence during in vivo infections remains to be investigated. However, as FIP viruses arise by mutations during FECV infections, strains such as UG-FH8 are probably more prone to the introduction of pathotype-switching mutations.

In contrast to betacoronaviruses, both alpha- and gammacoronaviruses lack a receptor-destroying enzyme that keeps the sialic acid binding site free from competitive inhibitors to ensure efficient interaction with cell surface sialic acids. For these viruses, including TGEV, PEDV and IBV, it has been described that their sialic acid binding capacity becomes more pronounced when virions are pretreated with NA (Park et al., 2010; Schultze et al., 1992, 1996). This masking effect has also been described previously for mammalian siglecs such as sialoadhesin (Delputte & Nauwynck, 2004) and CD22 (Razi & Varki, 1998), and was also demonstrated for FECV in the present study. The effect of unmasking the viral sialic acid binding activity on viral infectivity has only been studied for TGEV. Whereas NA treatment of the virus enhanced sialic acid-mediated attachment to cells (Schwegmann-Wessels et al., 2002), it had no effect on the infectivity of the viruses (Schultze et al., 1996). In contrast to TGEV, removal of sialic acids from FECV virions had an enhancing effect on the viral infectivity in vitro. By using sialyllactoses as competitive inhibitors, it was shown that this was due to an enhanced sialic acid binding (2,6-α-linked over 2,3-α-linked). By performing competitive inhibition experiments with the highly 2,6-α-sialylated macromolecule BSM (Tsuji & Osawa, 1986), which in contrast to sialyllactose allows multivalent binding, replication of both untreated and NA-treated viruses was inhibited almost completely at a concentration of 1 mg ml⁻¹. This inhibition was not seen with another mucin, PGM, which especially contains neutral and sulphated oligosaccharides (Nordman et al., 1997). These results indicate that both untreated and NA-treated viruses use sialylated compounds as attachment factors in the enterocyte cultures. However, when sialic acid binding is reduced/avoided by desialylation of cells, the viral receptor can be more efficiently reached, resulting in an enhanced infectivity of both untreated and NA-treated viruses. In addition, viruses become less susceptible to competitive inhibition with sialylated compounds in desialylated cells (sialyllactoses had no effect and the effect of BSM was less pronounced compared with untreated cells), showing that sialic acid binding is not a prerequisite

Fig. 4. Effect of BSM on the replication of untreated and NA-treated viruses in both untreated and desialylated cells. Both untreated and NA-treated virions were pre-incubated with different concentrations of BSM for 30 min at 37 °C before inoculation. At 1 h p.i., the inoculum was removed by three washings and the relative percentage of infected cells was assessed 12 h p.i. Data are expressed as the mean ± SD of the results of four separate experiments. Significant differences (P < 0.05) are indicated with an asterisk.
for the initiation of FECV infections in vitro. This can explain why NA treatment of the virus is detrimental in desialylated cells, as its enhanced sialic acid binding activity potentially delays the virus in its receptor engagement by binding to remaining sialic acid residues. This decay activity of sialic acids in vitro can be explained by the fact that FECV has no receptor-destroying activity mediating its detachment from non-receptor glycoproteins. Although the lack of a receptor-destroying enzyme seems to disadvantage the virus, allowing competitive inhibitors to cover the virus is possibly a major strategy of these viruses to enable intestinal infections. In contrast to non-enveloped viruses, which represent the majority of all enteritis-inducing viruses, coronavirus are more prone to inactivation by different unfavourable conditions and it is still unclear how the enteritis-inducing coronaviruses survive the harsh conditions (low pH, enzymes and bile salts) in the gastrointestinal tract. As sialic acids can confer protection against enzymatic degradation (Schauer, 2000), additional covering with sialylated compounds such as mucins can help the virus to survive the unfavourable conditions in the upper part of the gastrointestinal tract (Schultze et al., 1996).

Based on the results of the present study, a hypothetical model for the initiation of FECV infections in vivo can be proposed. In addition to the abundant intrinsic glycosylation (Siddell et al., 1983), FECV most probably becomes covered with sialylated compounds encountered during exit from infected host cells and/or during uptake in the oral cavity. This protects the virus and potentially masks the viral sialic acid binding site, allowing the virus to pass through the stomach without degradation or distraction by attachment to gastric mucins. However, during this passage viruses are faced with acidic environments and host/bacterial sialidases, which mediate hydrolysis of sialic acids, resulting in the release of sialic acids from the viral surface. This induces the liberation of the virus’ sialic acid binding domain, allowing FECV to escape from the intestinal flow by attaching to the mucus and to engage with its functional receptor on the enterocyte membrane. As FECV lacks its own receptor-destroying enzyme, passage through this mucus layer and efficient receptor engagement most likely depend on intestinal sialidases.

For TGEV, it has been shown that its enterotropism is highly dependent on its sialic acid binding capacity (Krempl et al., 1997). The sialic acid binding activity of FIP virus and the role of sialic acid binding in the enterotropism of FCoV remain to be investigated. In addition, it remains elusive if this sialic acid binding is also involved in further steps of the pathogenesis and the onset of FIP. In the present study, it was shown that sialylated compounds can act as inhibitors for at least FECV infections. This decay activity was also suggested by Paltrinieri et al. (2008), who proposed that the cat’s own sialylated acute-phase protein AGP can confer protection against the development of FIP. Whereas cats with FIP tend to have elevated, but hyposialylated serum AGP concentrations (Ceciliani et al., 2004), healthy cats in the same environment have hypersialylated AGP (Paltrinieri et al., 2008). This hyposialylation of AGP in FIP cats included both 2,6-α- and 2,3-α-linked sialic acids (Ceciliani et al., 2004). However, in contrast to the more pronounced decay activity of 2,6-α-linked sialic acids observed in the present study, 2,3-α-linked sialic acids on AGP seemed to be more involved in the determination of the outcome of FCoV infections (Ceciliani et al., 2004; Paltrinieri et al., 2008). As only FECV was considered in the present study, it would be interesting to investigate if changes in viral sialic acid binding activity occur during the pathotype switch.

In conclusion, this study shows that serotype I FECVs have a sialic acid binding capacity that is partially masked by virus-associated sialic acids. However, binding to terminal sialic acid residues on the enterocyte surface is not a prerequisite for infection and these sialic acids even seem to hamper efficient receptor engagement during in vitro infections. Nevertheless, if the in vivo situation is taken into account, the rationale for such a lectin binding capacity is more clear, as it gives the virus advantages in its confrontation with the harsh conditions and mucosal barriers in the intestinal tract. These insights provide new opportunities for antiviral intervention.

**METHODS**

**Viruses and cells.** Two serotype I FECV strains, UCD and UG-FH8, were propagated in feline colonocyte cultures in FBS-depleted medium and third passage strains were used for all infection experiments. All experiments were performed in both feline ileocyte and colonocyte cultures. Cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 Nutrient Mixture (1/1) supplemented with 100 U penicillin ml⁻¹, 0.1 mg streptomycin ml⁻¹, 0.1 mg gentamicin ml⁻¹, 5% FBS (Gibco-BRL) and 1% non-essential amino acids 100 × (Gibco-BRL). The origin of the viruses and cells has been described previously (Desmarets et al., 2013).

**NA treatment of cells.** To remove sialic acids from the enterocytes, monolayers of continuous ileocytes and colonocytes were washed two times with warm Ca²⁺- and Mg²⁺-enriched PBS. Then, cells were incubated with 50 mU NA ml⁻¹ from Vibrio cholerae (Roche Diagnostics) in Ca²⁺⁻ and Mg²⁺⁻-enriched PBS. Cells that were not treated with NA were incubated in Ca²⁺⁻ and Mg²⁺⁻-enriched PBS, and underwent the same manipulations as the NA-treated cells. After 1 h at 37°C, cells were washed three times with medium to remove the NA. Viability of the cells was assessed by ethidium monoazide bromide (EMA) staining, ensuring >99% viability with the used NA concentration.

**NA treatment of viruses.** To remove sialic acids from the virus, virus suspensions were incubated on a shaker for 1 h at 37°C with 50 mU GlycoCleave NA (Vibrio cholerae) enzyme beads ml⁻¹ (GALAB Technologies) in Ca²⁺⁻ and Mg²⁺⁻-enriched PBS. Beads were washed two times with Ca²⁺⁻ and Mg²⁺⁻-enriched PBS before incubation with the virus to remove buffers. Before inoculation, NA beads were separated from the virus by centrifugation (200 g, 10 min, 4°C). Untreated virions were incubated in Ca²⁺⁻ and Mg²⁺⁻-enriched PBS without beads, and underwent the same manipulations as the NA-treated virus.

**Infection experiments.** Cells were inoculated with either NA-treated or untreated virus (10⁵.⁸ and 10⁵.⁹ TCID₅₀ ml⁻¹ for FECV UCD and UG-FH8, respectively). After 5 or 60 min at 37°C, the unbound virus
particles were removed by three washing steps with medium and the cells were further incubated in medium for 12 h (37 °C, 5% CO₂). Then, cells were fixed with 4% paraformaldehyde for 10 min at room temperature.

**Immunofluorescence staining of infected enterocytes.** Cells were permeabilized with 0.1% Triton X-100 for 2 min at room temperature. Then, cells were incubated for 1 h at 37 °C with the monoclonal anti-nucleocapsid antibody 10A12 (produced and characterized in the laboratory of the authors) containing 10% normal goat serum, followed by incubation with goat anti-mouse FITC-labelled antibodies (Molecular Probes) for 1 h at 37 °C. Nuclei were stained with Hoechst 33342 (Molecular Probes) for 10 min at room temperature. Slides were mounted using glycerine–PBS solution (0.9 : 0.1, v/v) with 2.5% 1,4-diazabicyclo[2.2.2]octane (Janssen Chimica) and analysed by fluorescence microscopy (DM B fluorescence microscope; Leica Microsystems).

**Infection inhibition assays.** PGM, BSM, fetuin, lactoferrin, lactose, d-galactose, N-acetyl-d-glucosamine and N-acetyl-d-galactosamine were purchased from Sigma-Aldrich; 2,3-α-sialyllactose and 2,6-α-sialyllactose were from Carbosynth. NA-treated and untreated viruses were pre-incubated with different concentrations of each compound for 30 min at 37 °C. These virus–compound mixtures were used to inoculate feline colonocyte cultures. After 1 h at 37 °C, unbound virus particles were removed by three washing steps with medium and the cells were further incubated in medium for 12 h. Then, cells were fixed with 4% paraformaldehyde and stained as described above. Viability of the cells was assessed by EMA staining, ensuring >99% viability with the used concentrations.

**Statistical analysis.** Experiments were independently repeated four times and results were compared with the Mann–Whitney U test. Statistical analysis was performed using Prism version 5.0c (GraphPad). P<0.05 was considered significantly different.

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