European Journal of Medicinal Chemistry 216 (2021) 113296

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Discovery of deoxyceramide analogs as highly selective ACER3 inhibitors in live cells

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ARTICLE INFO

Article history:
Received 6 November 2020
Received in revised form
27 January 2021
Accepted 12 February 2021
Available online 24 February 2021

Keywords:
Sphingolipid
Ceramide
Ceramidase
Inhibitor
Therapeutic target
Enzyme activity

ABSTRACT

Acid (AC), neutral (NC) and alkaline ceramidase 3 (ACER3) are the most ubiquitous ceramidases and their therapeutic interest as targets in cancer diseases has been well sustained. This supports the importance of discovering potent and specific inhibitors for further use in combination therapies. Although several ceramidase inhibitors have been reported, most of them target AC and a few focus on NC. In contrast, well characterized ACER3 inhibitors are lacking. Here we report on the synthesis and screening of two series of 1-deoxy(dihydro)ceramide analogs on the three enzymes. Activity was determined using fluorogenic substrates in recombinant human NC (rhNC) and both lysates and intact cells enriched in each enzyme. None of the molecules elicited a remarkable AC inhibitory activity in either experimental setup, while using rhNC, several compounds of both series were active as non-competitive inhibitors with K_i values between 1 and 5 μM. However, a dramatic loss of potency occurred in NC-enriched cell lysates and no activity was elicited in intact cells. Interestingly, several compounds of Series 2 inhibited ACER3 dose-dependently in both cell lysates and intact cells with IC_{50}'s around 20 μM. In agreement with their activity in live cells, they provoked a significant increase in the amounts of ceramides. Overall, this study identifies highly selective ACER3 activity blockers in intact cells, opening the door to further medicinal chemistry efforts aimed at developing more potent and specific compounds.

1. Introduction

Besides playing structural roles, sphingolipids are acknowledged as a family of bioactive lipids. Ceramides are the central molecules in sphingolipid metabolism. They can be generated *via* three different pathways, the *de novo* pathway, hydrolysis of complex sphingolipids, and the salvage pathway from sphingosine 1-phosphate [1]. The canonical *de novo* pathway begins with the condensation of palmitoyl CoA with L-serine catalyzed by serine palmitoyltransferase. Mutations in this enzyme induce a permanent shift in the substrate specificity of the enzyme from L-serine to L-alanine and glycine, resulting in increased production of 1-deoxysphingolipids [2].

After their synthesis, ceramides are converted to complex sphingolipids (sphingomyelin, ceramide 1-phosphate and glycosphingolipids) by substitution at the C1-hydroxyl group with polar groups. Due to the absence of this function, the addition of a head group to form complex sphingolipids is precluded in 1-deoxysphingolipids. Moreover, while ceramides are degraded to ethanolamine phosphate and a fatty aldehyde via sequential hydrolysis of ceramides, phosphorylation of the resulting long chain

Abbreviations: AC, acid ceramidase; ACER3, alkaline ceramidase 3; ASAH2, neutral ceramidase gene; C6-urea-ceramide, D-*erythro*-*N*-[2-(1,3-dihydroxy-4*E*-octadecene)]-*N*′-hexane-urea; D-e-DMAPP, (1*S*,2*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol; LC/MS, liquid chromatography coupled to mass spectrometry; MEF, mouse embryonic fibroblasts; NC, neutral ceramidase; rhNC, recombinant human NC.

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https://doi.org/10.1016/j.ejmech.2021.113296
0223-5234/

base to the corresponding phosphate and final irreversible retro-aldolic cleavage by sphingosine-1-phosphate lyase [1], this pathway is not possible for 1-deoxysphingolipids, as the essential catabolic intermediate, sphingosine 1-phosphate, cannot be formed from the 1-deoxybases [2].

Hydrolysis of ceramides occurs by the action of ceramidases, which are encoded by five known genes and are distinguished by the pH required for optimal activity (acid ceramidase (AC, ASAH1), neutral ceramidase (NC, ASAH2) and alkaline ceramidases 1, 2 and 3 (ACER1, ACER2 and ACER3) [3]. Conversely, 1-deoxy(dihydro) ceramides are exclusively hydrolyzed by AC [4]. Amongst the five different enzymes, AC, NC and ACER3 are the most ubiquitous and their therapeutic interest as targets in cancer diseases has been well sustained [5].

AC is a glycosylated 50 kDa enzyme belonging to the N-terminal nucleophile (Ntn) superfamily of hydrolases. It is synthesized in the ER as an inactive proenzyme that is activated in the lysosome through autocleavage, rendering a mature heterodimeric enzyme containing α- and β-subunits [6]. The crystal structures have shown that the catalytic center is buried in the proenzyme, while autocleavage triggers a conformational change exposing the active site hydrophobic channel [7]. Genetic loss-of-function mutations in AC are the underlying cause of two severe rare diseases: Farber disease and spinal muscular atrophy with progressive myoclonic epilepsy [8]. Furthermore, changes in the expression levels of AC has been found to be relevant in melanoma [9–12], prostate cancer [13–16] and acute myeloid leukemia [17–19].

NC is a single transmembrane domain glycoprotein highly expressed in the small intestine and colon and it appears to regulate the levels of bioactive sphingolipid metabolites in the intestinal tract [20]. Its crystal structure revealed a catalytic domain, a short linker, and an immunoglobulin-like domain. The structure also revealed that the active site of human NC is composed of a narrow, 20 Å deep, hydrophobic pocket with a Zn^{2+} ion at the bottom [21]. Besides a few other roles [22–26], a function of NC in cancer is supported by the findings that NC downregulation is involved in gemcitabine-induced growth suppression [27] and by recent studies showing that NC regulates cell survival in colon cancer cells [28,29]. These studies demonstrated that inhibition of NC in a xenograft model delayed tumor growth and that mice lacking NC were protected from azoxymethane-induced tumor formation [28].

ACER3 is a seven-transmembrane domain with an intracellular N-terminus exposed to the cytoplasm and the C-terminus facing the lumen of the endoplasmic reticulum or Golgi apparatus. The protein contains functionally connected catalytic Zn^{2+} and Ca^{2+}-binding sites, providing a structural explanation for the known regulatory role of Ca^{2+} on ACER3 enzymatic activity [30,31]. Genetic ACER3 deficiency leads to progressive leukodystrophy in early childhood [32], a rare disease for which no treatment is available. Furthermore, ACER3 has been reported to contribute to hepatocellular carcinoma [33] and acute myeloid leukemia pathogenesis [34] and its downregulation has been shown to inhibit cell proliferation [35].

The role of ceramidases in human diseases is gaining increasing attention. However, current research in this field has been hampered by the lack of specific inhibitors for some of these enzymes. Although several inhibitors of ceramidases have been discovered [36], most of them target AC. Over the last two decades, a number of NC inhibitors have been reported, but their potency is low, their specificity has not been thoroughly investigated and none has reached clinical effect [36]. A recent article reports on a large (>650,000 small molecules) high-throughput screening assay for NC inhibitors that has resulted in interesting leads [37]. Unfortunately, no chemical structures are provided and further investigation of hits in cell free systems and intact cells has not been performed. On the other hand, only one inhibitor of alkaline ceramidases (ACER) has been reported, namely (1*S*,2*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol (D-e-DMAPP) [38], but the specific ACER target has not been identified and a few articles claim that D-e-DMAPP inhibits also AC [39] and NC [40]. Thus, the identification of new tools should significantly advance our knowledge on the function of the different ceramidases, allow a better understanding of their role in the regulation of biological processes and in addition, may potentially lead to novel therapeutic strategies.

From the biological stand point, 1-deoxysphingolipids are relevant in diseases such as hereditary sensory and autonomic neuropathy, a genetic condition caused by mutations in serine palmitoyltransferase (see above). Furthermore, several clinical studies showed that 1-deoxysphingolipids are altered the metabolic syndrome and in type 2 diabetes [41]. Moreover, plasma 1-deoxysphingolipids are also elevated in von Gierke disease [42], and appear to be also relevant in the progress from hepatosteatosis to steatohepatitis [2].

In the light of these overall precedents, we envisioned that 1-deoxysphingolipid analogs might provide compounds with interesting biological properties. Herein, we report on the screening of 1-deoxysphingolipid analogs (Fig. 1) on AC, NC and ACER3 that yield to the identification of highly selective ACER3 inhibitors acting both in lysates and intact cells.

2. Materials and methods

2.1. Chemical synthesis

Synthesis and characterization of compounds is detailed in the supplementary materials.

2.2. Cell culture

The A375 cell line stably overexpressing ASAH1 under the control of a doxycycline-responsive promoter was kindly provided by Dr. Carmen Bedia and Prof. Thierry Levade. HT29 cells were obtained from ATCC, Farber disease (FD) cells transformed to stably overexpress AC (FD10X) were a kind gift of Prof. Jeff Medin and *ASAH2*$^{(-/-)}$ mouse embryonic fibroblasts (MEF) were kindly provided by Prof. Richard Proia. In the latter, experiments were conducted at least two weeks after thawing. All cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum). Cells were kept at 37 °C and 5% CO_2 and routinely grown up to 70% confluence.

2.3. Cell transfection

The antibiotic selection in the A375 cell line stably overexpressing ASAH1 under the control of a doxycycline-responsive promoter was performed with blasticidin (3 μg/mL) and hygromycin B (250 μg/mL). Ectopic expression of AC was induced with doxycycline at 1 μg/mL for 24 h before use.

Transient transfections with the ASAH2-containing plasmid were performed with Lipofectamine® 2000 (Invitrogen) in a 12-well plate format. One day before transfection, $3x10^5$ cells/mL were plated in growth medium without antibiotics, so that cells would be 90–95% confluent at the time of transfection. For each transfection sample, cDNA was diluted in 50 μL of Opti-MEM®I without serum; lipofectamine (2 μL) was diluted in 50 μL of Opti-MEM®I Medium and incubated for 5 min at 25 °C. Next, both solutions were combined, mixed gently, and incubated for 20 min at 25 °C. The resulting solution was mixed with 400 μL of medium and it was added to cells. After 4 h at 37 °C and 5% CO_2, 1 mL of medium

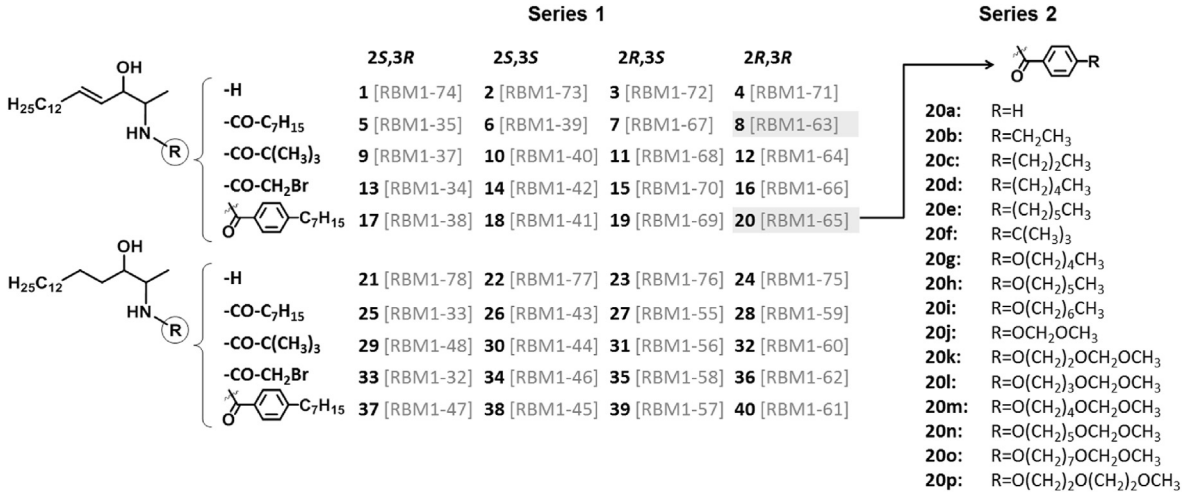


Fig. 1. Chemical structures of the 1-deoxyceramides studied in this work. Internal codes are shown in grey between brackets.

containing 10% FBS and tetracycline (10 ng/mL) was added. Cells were incubated for 24 h prior to testing for transgene expression and compounds addition for inhibitory activity determination.

2.4. Cell viability

Cells (0.1-0.25x10^6/ml) were seeded in 96 well plates (0.1 ml/well) and grown for 24 h. Cell viability was examined in triplicate samples by the MTT method after treatment with the indicated compounds or with the corresponding percentage of vehicle ($\leq$0.25% ethanol)

2.5. Fluorogenic ceramidase activity assay in vitro

Experiments with rhNC (R&D Systems, >95% pure) were carried out with 5 ng of protein (MW = 83 KDa) in 0.1 mL of buffer (see below), which affords a protein concentration of 0.6 nM for a substrate concentration of 20 μM. These conditions (substrate concentration much larger than protein concentration) are appropriate to use the Lineweaver-Burk plot to study the enzyme kinetics.

To prepare the lysates, cell pellets were resuspended in the appropriate volume of a 0.25 M saccharose solution containing the protease inhibitors aprotinin (1 mg/mL), leupeptin (1 mg/mL) and PMSF (100 mM). The suspension was submitted to three cycles of a 5 s sonication (probe) at 10 W/5 s resting on ice. The cell lysate was centrifuged at 600 *g* for 5 min. The supernatant was collected, and protein concentration was determined with BSA as a standard using a BCA protein determination kit (Thermo Scientific) according to the manufacturer's instructions.

All the enzyme activity assays were carried out in 96-well plates at a final volume of 100 μL/well. Reaction buffers were: 25 mM sodium acetate buffer pH 4.5 (AC), 25 mM phosphate buffer 150 mM NaCl 1% sodium cholate (NaChol) pH 7.4 (NC) and 50 mM HEPES 1 mM $CaCl_2$ pH 9 (ACERs). The reaction mixtures contained 25 μL/well of protein (5 ng recombinant NC or 25 μg cell lysate), 70 μL/well of substrate (prepared from 4 mM stock solutions in ethanol) and 5 μL/well of inhibitor (tested at concentrations indicated in the figure legends, prepared from 10 mM stock solutions in ethanol). The following fluorogenic substrates were used at the concentrations specified in the figure legends: for AC, RBM14C12 [43]; for NC, RBM14C24:1 [44] and for ACER, RBM14C16 [45]. Reaction mixtures were incubated at 37 °C for 1 h (recombinant NC) or 3 h (cell lysates). In all cases, reactions were stopped with 25 μL/well of MeOH and then 100 μL/well of $NaIO_4$ (2.5 mg/mL in 100 mM glycine-NaOH buffer, pH 10.6) was added. After incubation at 37 °C for 1 h in the dark, 100 μL/well of 100 mM glycine-NaOH buffer (pH 10.6) was added and fluorescence was measured spectrophotometrically at excitation and emission wavelengths of 355 and 460 nm, respectively. The same reaction mixtures without enzymes were used as blanks.

2.6. Fluorogenic ceramidase activity assay in intact cells

To determine ceramidase activity in intact cells, 2x10^4 cells/well were seeded in 96-well plates 24 h prior to the assay and maintained at 37 °C and 5% CO_2. Medium was replaced by 100 μL of fresh medium (DMEM 10% FBS) containing 10 μM of the corresponding RBM14 substrate and different concentrations of the indicated test compounds. The plate was incubated for 3 h at 37 °C in 5% CO_2. The reaction was stopped with 25 μL/well of MeOH and then 100 μL/well of $NaIO_4$ (2.5 mg/ml in glycine-NaOH buffer, pH 10.6) were added. After incubation at 37 °C for 1 h in the dark, 100 μL/well of 100 mM glycine-NaOH buffer (pH 10.6) were added and fluorescence was measured spectrophotometrically at excitation and emission wavelengths of 355 and 460 nm, respectively. The same reaction mixtures without cells were used as blanks. The following fluorogenic substrates were used at the concentrations specified in the figure legends: for AC, RBM14C12 [43] and for NC and ACER3, RBM14C16 [45]. Both substrates and test compounds were added simultaneously to the cell culture.

2.7. Lipid analysis

Cells were seeded at 0.2x10^6 cells/mL in 6 well plates (1 mL/well). After 24 h, medium was replaced with fresh medium containing either the treatment of interest or the corresponding vehicle as a control. After the indicated incubation times, medium was removed and cells were washed with PBS. Cells were collected by trypsinization. Ten μL of the cell suspension were used to count cells for each sample. Sphingolipid extracts, fortified with internal standards (*N*-dodecanoylsphingosine, *N*-dodecanoylglucosylsphingosine, *N*-dodecanoylsphingosylphosphorylcholine, C17-sphinganine (0.2 nmol each) were added and lipids were analyzed by LC/MS with the reported equipment and instrument conditions [46].

3. Results

3.1. Synthesis

Due to the interesting properties, biological activity and improved metabolically stability of 1-deoxysphingolipids, as well as to the finding that 1-deoxydihydroceramides with the natural stereochemistry are exclusively hydrolyzed by AC [4], a small library of derivatives was prepared based on the structure of 1-deoxysphingosine and 1-deoxysphinganine covering the four possible stereoisomers. The *N*-acyl moieties were chosen because of our previous reports showing that the corresponding (dihydro) ceramides are ceramidase inhibitors [15,47]. Sphingoid bases were prepared as previously reported [48] and deoxyceramide analogs (Fig. 1) were synthesized by amidation of the proper enantiopure sphingoid base with the corresponding acid derivative according to the previously described methodology (Scheme 1).

3.2. Studies in recombinant protein and cell lysates

Given the interest of AC, NC and ACER3 as therapeutic targets in cancer, Series 1 was first screened over these three enzymes at equimolar concentrations with the appropriate substrates. The activity over AC was tested in lysates from AC-overexpressing cells using RBM14C12, as the best AC fluorogenic substrate [43]. As shown in Figure S1, none of the compounds exhibited a remarkable inhibition of AC, even when the inhibitor/substrate molar ratio was increased to (2:1) (data not shown). Intriguingly, the bromoacetyl derivatives of 1-deoxysphingosine and 1-deoxysphinganine with the natural configuration (**13** and **33**, Fig. 1) did not inhibit AC, in contrast to their analogs containing the C1−OH, which are potent activity based irreversible AC inhibitors [15,49].

Activity over NC was initially tested using recombinant human NC (rhNC) and RBM14C16, as the best NC fluorogenic substrate [45]. Two compounds, **8** and **20**, completely blocked NC activity when tested at a substrate/inhibitor ratio of 1:1 (20 μM) and provoked a 70 and 90% inhibition, respectively, at 5 μM (20 μM substrate) (data not shown). Reducing **8** and **20** concentrations to 1 μM (substrate/inhibitor molar ratio of (20:1)) provoked a 50% and 70% inhibition of NC, respectively (Figure S1). It is worth noting that the two compounds are 1-deoxyceramides, while the corresponding saturated analogs were inactive at inhibiting NC, indicating that the C4-double bond is required for inhibition. Furthermore, they have a (2*R*,3*R*) configuration, different from that of the natural substrate (2*S*,3*R*), which agrees with a non-competitive inhibition where the inhibitor binds the enzyme at allosteric sites (see below).

Next, compounds were screened against ACER3 using lysates of *ASAH2*-null (*ASAH2*$^{(-/-)}$) mouse embryonic fibroblasts (MEF). These cells are considered a suitable model to screen ACER3 inhibitors since they lack NC, and ACER3 is the only alkaline ceramidase detected [45]. No significant ACER3 inhibition was elicited by these compounds at basic pH, in the presence of Ca^{2+} and at a substrate/inhibitor molar ratio of (1:1) (Figure S1).

Based on the structure of one of the most potent compound identified (**20**) as NC inhibitor, a focused library of analogs was next prepared including a set of more hydrophilic derivatives (Table S1) by modification of the benzene *para*-substituent by either shortening the chain length or introducing oxygen atoms. In this case, O-alkylbenzoic acids bearing a alkyl/polyoxygenated side chain were obtained by coupling of *p*-hydroxybenzoic acid methyl ester with the respective halogen derivatives using a two-step sequence (condensation and saponification) (Scheme 1).

Compounds belonging to this Series 2 were again screened against AC, NC and ACER3. None of the compounds inhibited AC at equimolar (Fig. S1) or two-fold molar (not shown) concentrations with the substrate (20 μM) when assayed in cell lysates from AC overexpressing cells. However, compounds **20** and **20l** were slightly inhibitory at 40 μM (Fig. 3A). Conversely, several inhibitors were also identified in the screening of Series 2 against recombinant NC, although none of them was more active than **20** (Fig. 2A). These results provided some insights into structure-activity relationships on the recombinant protein. Hence, decreasing the *p*-alkylphenyl substituent chain length (**20a**, **20b** and **20c**) resulted in reduction of the inhibitory activity, as compared to **20**, while compounds **20d** and **20e** had a similar activity to the reference compound. Introduction of one oxygen atom in the *p*-alkylphenyl substituent afforded compounds with similar inhibitory activity, while addition of two additional oxygen atoms resulted in reduction of activity (compare **20k** *vs* **20g**; **20l** *vs* **20h**, and **20m** *vs* **20i**). In addition, changing the oxygen position along the chain had also a negative effect on NC inhibitory activity (compare **20l** *vs* **20p**). Interestingly, the screening of the focused library on ACER3 also identified some inhibitors, mostly corresponding to the ω-methoxymethoxy family (Fig. 2B). Furthermore, activity against ACER3 was shown to be dose dependent for the two compounds examined (**20l** and **20m**) (Fig. 3C).

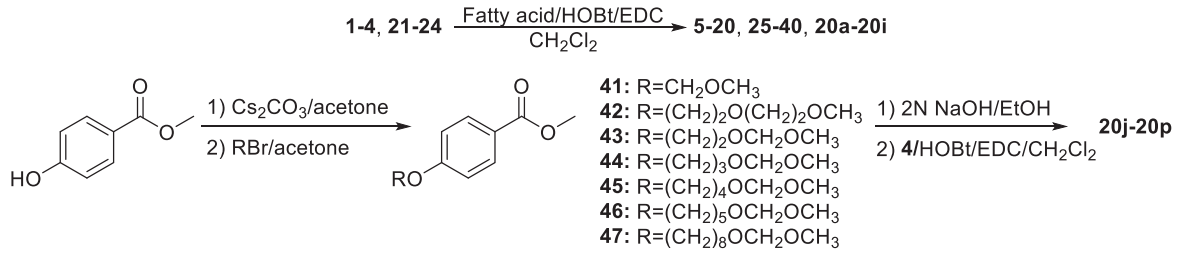
To get a deeper insight into the mode of inhibition against NC and ACER3, kinetic experiments were then conducted with **8**, **20, 20l and 20m**, two of the identified NC/ACER3 inhibitors[2]. Incubation of rhNC with different amounts of inhibitor at varied substrate concentrations resulted in modified V_{max} without significant changes in the K_m values (Fig. 4A), indicating that compounds **8**, **20**, **20l** and **20m** were non-competitive inhibitors of NC with K_i values of 2.0, 0.8, 5.4 and 3.6 μM, respectively. A similar outcome was observed for the two compounds active on ACER3, which behaved also as non-competitive inhibitors with apparent K_i values of 25.5 μM (**20l**) and 34.3 μM (**20m**) (Fig. 4B).

To compare the effects observed on ACER3 containing lysates (Fig. 2B), the inhibitory activity of **8**, **20**, **20l** and **20m** was then tested in lysates of HT29 cells transiently transfected to overexpress NC (Fig. 3B). A specific NC substrate, bearing a nervonic acid amide (RBM14C24:1) was employed in this case, thereby enabling only the measurement of NC activity [44]. Surprisingly, only **20l** and **20m** were shown to dose-dependently inhibit the hydrolysis of RBM14C24:1 (10 μM) at neutral pH (Fig. 3B) whereas no inhibition was observed with **8** and the parent compound **20**. Moreover, a remarkable reduction in potency was found in cell lysates, as compared to the recombinant enzyme, since a >40 μM concentration was necessary to achieve a 50% inhibition (Fig. 3B). A similar outcome occurred with the C6-urea-ceramide (D-*erythro*-*N*-[2-(1,3-dihydroxy-4*E*-octadecene)]-*N*′-hexane-urea-sphingosine), a reported NC inhibitor [50], which inhibited rhNC and NC from cell lysates with IC_{50}'s of 18 nM and 366 nM, respectively (Fig S2A). This decrease of potency observed when using cell lysates as compared to recombinant purified protein suggest that caution should be taken when interpreting data obtained with recombinant NC protein.

3.3. Studies in intact cells

Since cell-based assays consider the biological complexity of the cell and include factors, such as uptake and concentration by intracellular compartmentalization, that might have a positive impact on both inhibitory potency and selectivity, the activity of **8**,

[2] With the idea of testing the compounds in intact cells, we chose compounds **20l** and **20m** as they have similar lipophilicity to that of cell permeable *N*-octanoyl-sphingosine (Table S1) [56,57] with the lowest reduction in NC inhibitory activity, as compared to **20**.



Scheme 1. Synthesis of compounds used in this study and shown in Fig. 1. The preparation of the free bases **1**–**4** and **21**–**24** was conducted as reported [48].

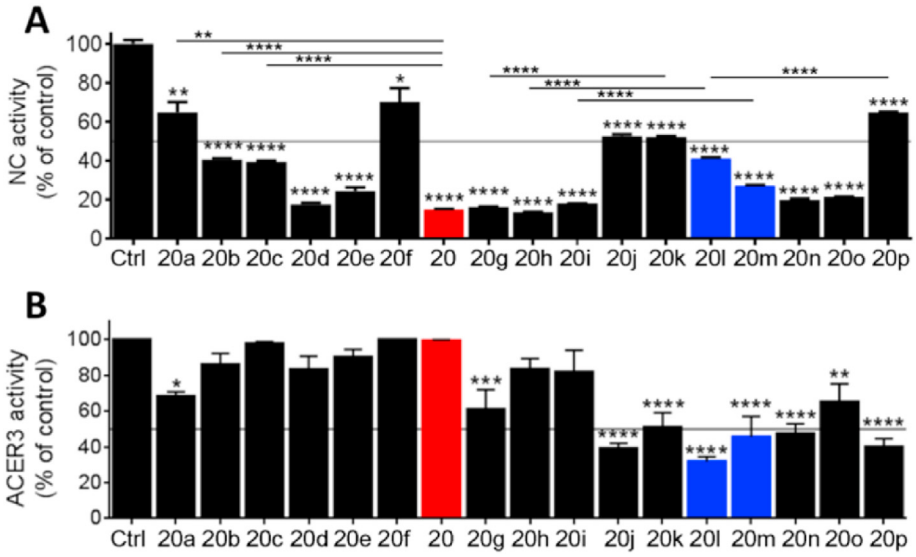


Fig. 2. Activity of Series 2 over rhNC (A) and ACER3 (ASAH2-null cell lysates) (B). Concentration of substrate was 20 μM (A) or 10 μM (B) and that of test compounds was 1 μM (A) and 20 μM (B). Suitable buffers and pH were used for each specific activity. Data (mean ± SD) were obtained from two to three experiments with triplicates. Asterisks above the SD bars denote statistical significance over controls at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, while statistical significance between specific groups is indicated with asterisks above lines (One-way ANOVA followed by Dunnett's multiple comparisons test).

20, **20l** and **20m** was next examined in intact cells. Like in the studies with cell lysates, test compounds and substrates were added simultaneously to the appropriate cell lines and incubations proceeded for 3 h. By using similar conditions, both in lysates and intact cells, we could get an estimation of the global effects of uptake and compartmentalization of the compounds on their inhibitory activity. However, a real analysis of both factors would require the use of fluorescent derivatives and the study of their subcellular distribution to confirm that they reach the organelles containing the target enzymes.

Activity over AC was tested using AC overexpressing A375 cells (A375/+Dox) and the fluorogenic substrate RBM14C12. No inhibition could be observed with **20,** whereas **20l** and **20m** exhibited very weak AC inhibitory activity (IC50's > 200 μM) (Fig. 5A).

Preliminary experiments to investigate the compounds' effect on NC revealed that they were very toxic in the presence of methyl-β-cyclodextrin, the co-solvent required for the administration of the specific fluorogenic NC substrate (RBM14C24:1) in intact cells [44]. Since the NC specific substrate could not be used, RBM14C16 (substrate for both NC and ACER3) was employed and the inhibition experiments were carried out in ASAH2-null MEF (ACER3) and their wild type counterparts (WT-MEF) (NC and ACER3). Data comparison should allow discerning between NC and ACER3 inhibitory activity. As shown in Fig. 5B and C, compounds **8** and **20** exhibited insignificant inhibition of either NC or ACER3 while **20l** and **20m**, otherwise, both inhibited the hydrolysis of RBM14C16, and thereby umbelliferone production, in both cell lines (Fig. 5C). Statistical comparison of the dose-response data revealed that the four curves and their corresponding IC_{50} values were not statistically different ($P = 0.2206$). As the combined inhibition of both ACER and NC should result in higher inhibition rates in WT-MEF, these results, showing similar activities in both WT-MEF (which contain NC and ACER3 activity) and *ASAH2*-null MEF (lacking the NC gene), support that NC inhibition by **20l** and **20m** in intact cells is negligible and that ceramidase inhibition observed in intact cells is solely due to their action over ACER3 in both cell lines.

To confirm the effect of these two compounds on intracellular ceramide levels, a lipidomic analysis was performed in *ASAH2*-null MEF and in AC overexpressing A375 cells treated with either **20l** or **20m**. To this end, compounds were administered to cells and lipids were extracted and analyzed (LC/MS) after 3 h of treatment. Gratifyingly, in agreement with the results found with the fluorogenic assays, a significant 1.5-2-fold increase in the total amounts of ceramides was provoked by both **20l** and **20m** in *ASAH2*-null MEF, but no effect could be detected on sphingosine, sphingomyelin and glucosylceramide levels (Fig. 6A). Regarding the ceramides species, all of them were elevated by treatment with the compounds over vehicle controls, except for C24:0-ceramide, which was unaffected (Fig. 6B). Conversely, only a small effect on ceramide levels was provoked by **20l** in AC-overexpressing A375-cells (Fig. S3).

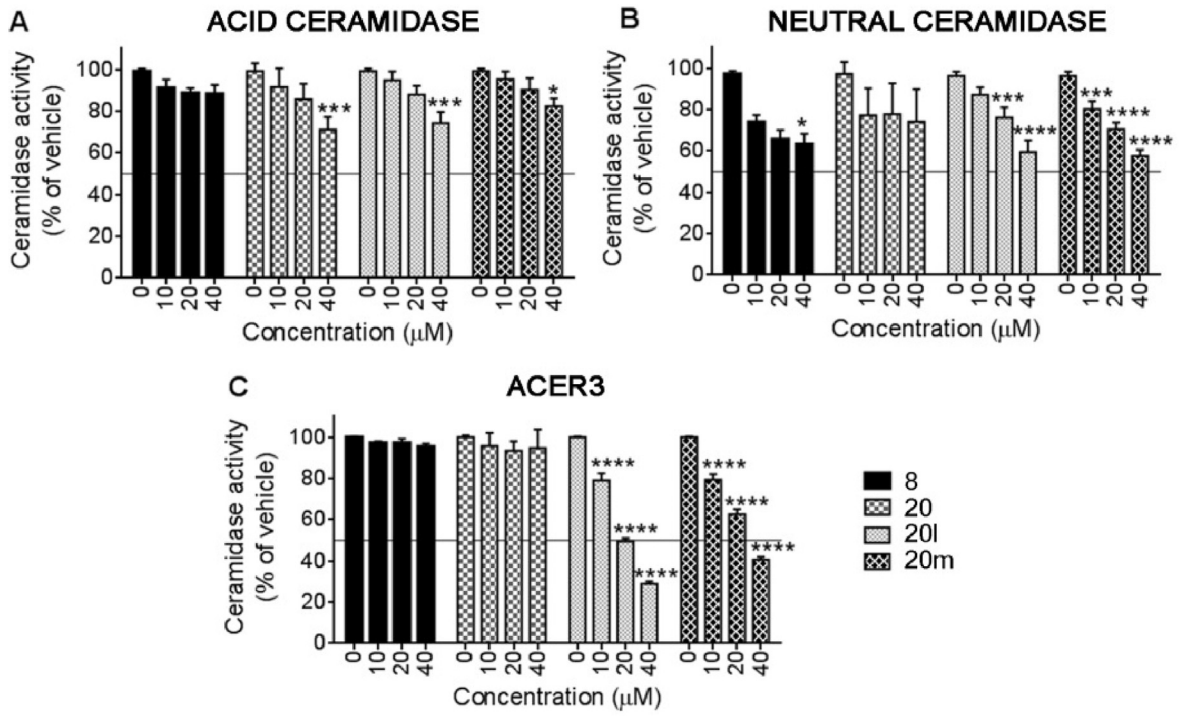


Fig. 3. Dose response of compounds on ceramidase activities in cell lysates. Experiments were carried out for 3 h (A, C) or 1 h (B) using 25 μg of lysates of AC overexpressing A375 cells (A), NC overexpressing HT29 cells (B) and ASAH2-null MEF (ACER3) (C), in 25 mM sodium acetate buffer, pH 4.5 (A), 25 mM phosphate buffer, 150 mM NaCl, 1% sodium cholate, pH 7.4 (B), or 50 mM HEPES 1 mM $CaCl_2$, pH 9 (C). Concentration of substrates (A, RBM14C12; B, RBM14C24:1; C, RBM14C16) was 10 μM. Data (mean ± SEM) was obtained from three to four experiments with triplicates. Asterisks denote statistical significance over controls at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (One-way ANOVA followed by Dunnett's multiple comparisons test).

4. Discussion

In our endeavour towards developing ceramidase inhibitors of pharmacological interest, two series of 1-deoxy(dihydro)ceramides were synthesized and screened against NC, AC and ACER3, the most ubiquitous ceramidases identified as therapeutic targets in cancer diseases [5]. Series 1 includes several diastereomeric *N*-acyl 1-deoxysphingosine and 1-deoxysphinganine amide derivatives, while Series 2 contains analogs of **20** with presumably improved bioavailability. An important feature of both libraries is their metabolic stability in front of enzymes acting at the C1—OH level to produce sphingomyelin, ceramide 1-phosphate, glycosphingolipids and the long chain base phosphates [51].

Whereas some compounds caused a significant and potent non-competitive inhibition of recombinant NC, studies performed in cell lysates with the most active inhibitors indicated a dramatic loss of potency, while several compounds of Series 2 inhibited ACER3. The identified selective ACER3 inhibitors, mostly belonging to the ω-methoxymethoxy family of Series 2, have a (2*R*,3*R*) configuration, different from that of the natural substrate (2*S*,3*R*), which suggests that the inhibitors bind the enzyme at allosteric sites in agreement with kinetic experiments showing that both **20l** and **20m** do not compete with the substrate active center.

This selective effect on ACER3 was further confirmed in studies with **20l** and **20m** using intact cells both in activity assays and after measurement of sphingolipid levels using mass-spectrometry methods, showing the expected increase in ceramides upon treatment with both compounds. However, no selective accumulation of long chain unsaturated ceramides was induced by **20l** and **20m**, which is at odds with reported data showing that ACER3 preferentially hydrolyzes these particular species [35] and that ACER3 upregulation prevents the buildup of C18:1-ceramide in mouse hepatocytes, while loss of ACER3 specifically augments C18:1-ceramide and C20:1-ceramide without affecting other ceramide species [52]. However, in another study showing that ACER3 is involved in aging-associated neurodegenerative disorders in humans [53], the effect of ACER3 knockout on the levels of ceramides and sphingosine was found to depend on both the age and the part of the brain examined, with the highest changes occurring in older tissues. However, no specific accumulation of long chain unsaturated ceramides was observed in ACER3 knockouts as compared to wild types.

On the other hand, neither **20l** nor **20m** provoked the expected reduction in the amounts of sphingosine. A similar outcome was observed in ACER3-knockout mouse hepatocytes [52] and in 6 week old brains of ACER3 knockout mice [53]. Conversely, the expected reduction in sphingosine [35] or its phosphate [33] was observed in other ACER3 knockout cell models. Overall, although C18:1-ceramide is clearly involved in some biological actions of ACER3, the effects of up- and downregulation of this enzyme on ceramide species and their catabolic products seem to depend on the cell type, the type of stimulus and the biological context.

It is worth noting that the three cell permeable compounds, namely **8**, **20l** and **20m**, were very toxic when tested in the presence of methyl-β-cyclodextrin, a cholesterol depleting agent, in HT29 cells overexpressing NC. A study reported by Romiti et al. [54] showed that endothelial cell treatment with cyclodextrin enhanced NC activity in caveolin-enriched membranes, indicating a negative role for cholesterol in NC regulation. It is possible that methyl-β-cyclodextrin removes cholesterol from membrane pools leading to NC activation, the inhibition of which by the compounds would lead to ceramide increases and subsequent cell death. However, ceramide analysis by LC/MS did not show the expected increase in cells treated with the compounds in the presence of methyl-β-

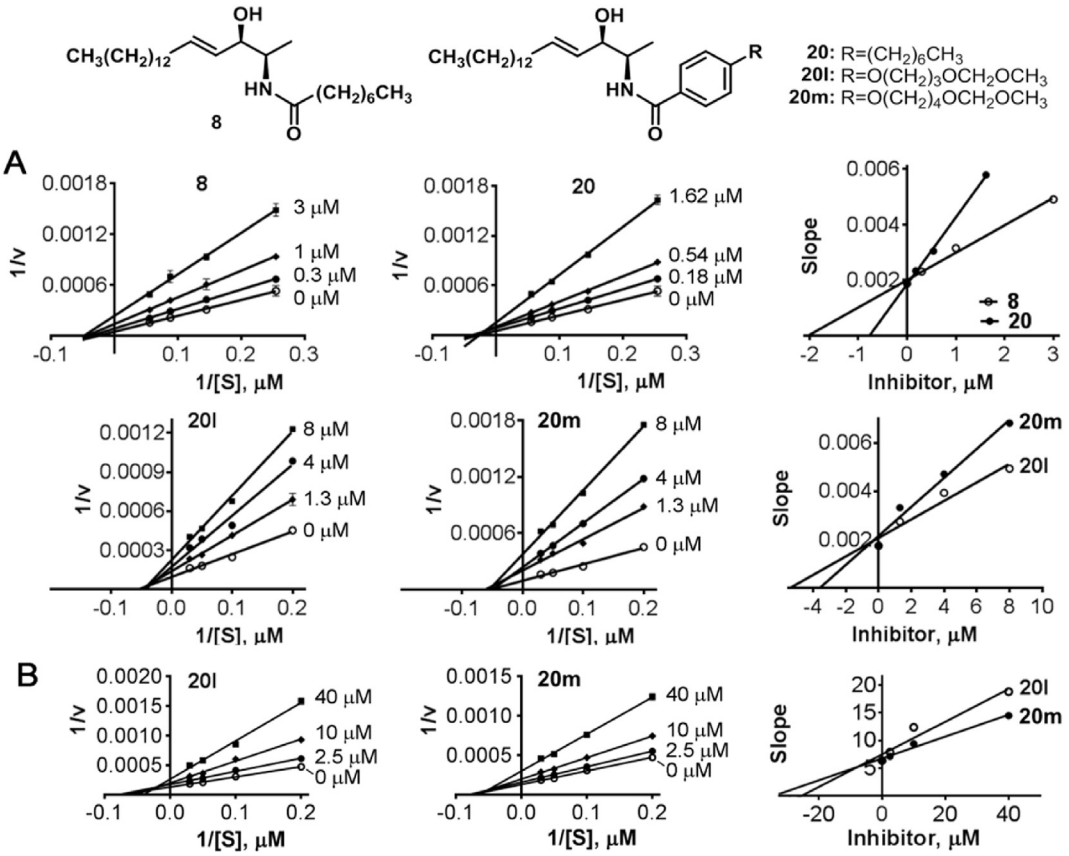


Fig. 4. Kinetics of inhibition of NC and ACER3 by **8**, **20**, **20l** and **20m**. Experiments were carried out using recombinant NC (5 ng, 0.6 nM) in 25 mM phosphate buffer, 150 mM NaCl, 1% sodium cholate, pH = 7.5 (A) or lysates from ASAH2-null MEFs (25 μg) in 50 mM HEPES 1 mM $CaCl_2$ pH 9 (B). Concentrations of substrate (RBM14C16) were 3.9, 6.9, 11.3 and 18 μM (**8** and **20**) and 5, 10, 20 and 40 μM (**20l** and **20m**). Data (mean ± SD) were obtained from four experiments with triplicates. K_i, as determined from the slope of the regression lines at each inhibitor concentration, were **8**, 2.0 μM; **20**, 0.8 μM; **20l**, 5.4 μM and **20m**, 3.6 μM in NC and **20l**, 25.5 μM and **20m**, 34.3 μM in ASAH2-null MEFs. Similar values were obtained using the x-intercept values.

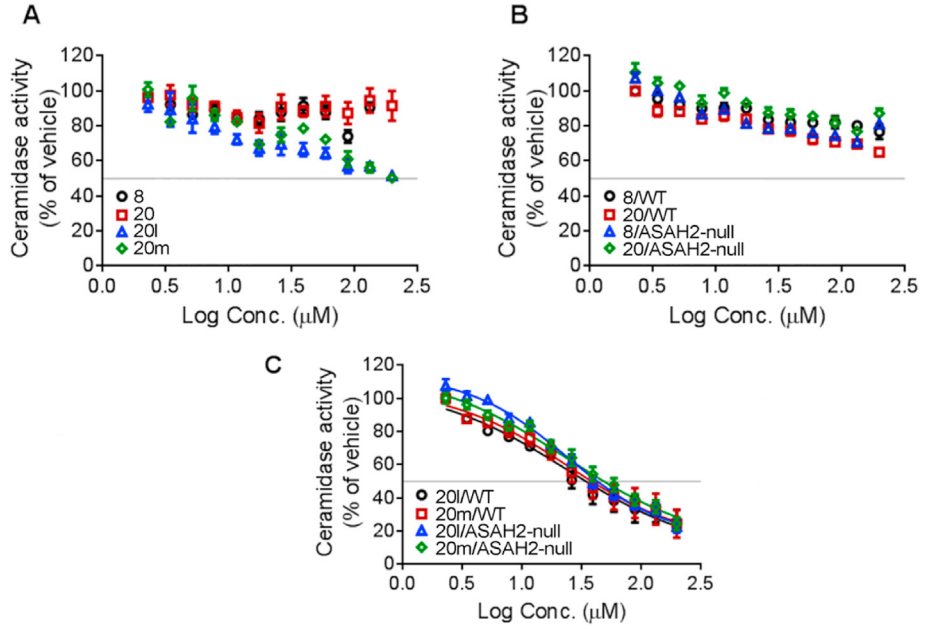


Fig. 5. Effect of inhibitors on umbelliferone production from fluorogenic substrates in intact cells. AC overexpressing A375 cells (A), WT-MEF (B,C) and ASAH2-null MEF (B,C) were incubated with the fluorogenic substrate (A, RBM14C12 (10 μM); B and C, RBM14C16 (10 μM)) and different concentrations of the specified compounds in DMEM 10% FBS for 3 h and enzyme activity was determined by the fluorogenic assay. Data were normalized by protein quantification (SRB assay, >90% cell viability). Data (mean ± SD) were obtained from three experiments with triplicates. In C, statistical comparison of fits concluded that the $LogIC_{50}$ was the same for all data sets ($p = 0.2206$) and the IC_{50} had a best fit value of 23.7 μM.

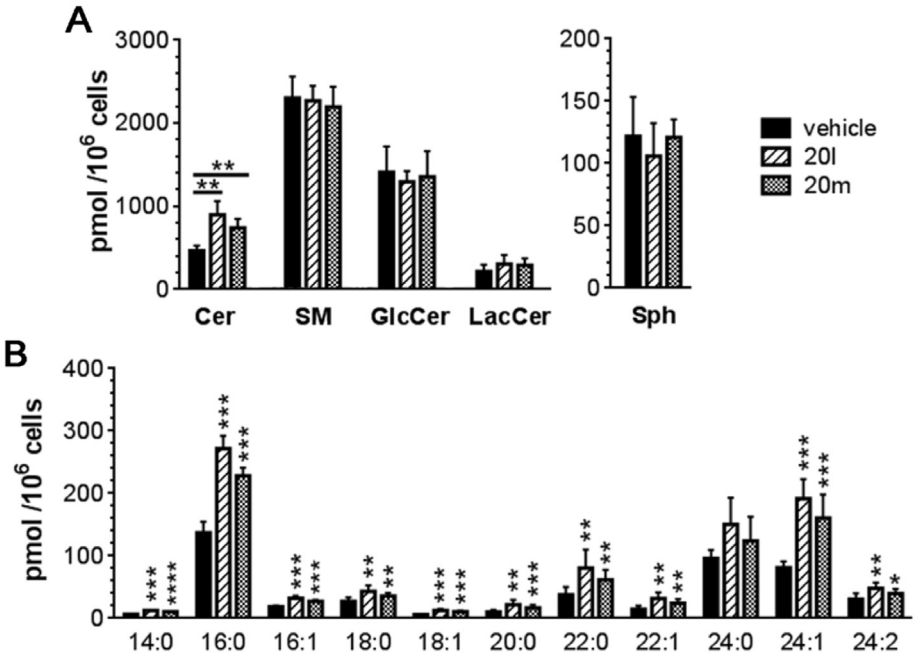


Fig. 6. Effect of inhibitors on sphingolipid levels in lysates of ASAH2-null MEF. Cells were treated with 50 μM of compound and vehicle (control) in DMEM 10% FBS. After 3 h, lipids were extracted and analyzed by LC/MS. Data (mean ± SD) were obtained from two experiments with triplicates. Asterisks indicate statistical significance over vehicle at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (unpaired, two-tailed *t*-test). Total lipids are shown in A and ceramides species are represented in B.

cyclodextrin (data not shown). This suggests that cell death is provoked by the compounds independently of their effect on ceramide metabolism and that cells are sensitized to the molecules by the disturbance of cell membranes induced by cholesterol depletion.

An additional important finding of this article is the remarkable decrease of potency experienced by the NC inhibitors when used in NC-overexpressing cell lysates as compared to rhNC. Importantly, such activity reduction also occurred with C6-urea-ceramide, a competitive NC inhibitor [50], indicating that it is not exclusive to the non-competitive inhibitors reported here. The reason for this potency loss may lie in the structural differences between the commercially available rhNC and the natural enzyme. While NC is bound to the plasma membrane with a single transmembrane helix [21], rhNC lacks a part of the *N*-terminus that includes a few aminoacids of the transmembrane domain (Fig. S4). The truncated protein is soluble in aqueous buffers and it is possible that the inhibitor binding domain conformation in rhNC has a higher affinity for the inhibitors than in the membrane bound enzyme. In this regard, as an example, removal of the membrane anchor in calnexin to create a soluble protein resulted in changes in the profile of substrate binding [55]. In their library screening, Otsuka et al. [37] used secreted NC purified from Sf9 insect cells [21]. Their protein corresponds to the extracellular region (residues 99–780) of human NC with a C-terminal hexahistidine tag and an N-terminal secretory signal. Whether the inhibitors discovered in that high throughput screen retain activity over the natural protein in cell free systems or in live cells has not been reported. This would be interesting to know, as in case of activity reduction, it would alert against the convenience of using genetically modified soluble NC in screening programs and would sustain that studies in cell lysates or preferably live cells must be conducted to avoid false expectations in terms of inhibitory potency on the natural, membrane bound enzyme.

Efforts of numerous groups over the last two decades have enabled the identification of several ceramidase inhibitors. However, in contrast to the better-characterized AC or NC, selective inhibitors of ACER3 are still lacking. Herein, we report that the screening of two series of deoxy(dihydro)ceramides against AC, NC and ACER3 resulted in the discovery of highly selective ACER3 inhibitors in live cells and discloses a scaffold for further medicinal chemistry efforts aimed at improving the ACER3 inhibitory potency. Such efforts should lead compounds devoid of off-target activities in cellulo as expected for **20l** and **20m** due to the high concentrations required for activity in the cell-based assay. Docking studies against the crystal structure of ACER3 [31] should aid in the design of the best ligands for further use in the study or treatment of diseases linked to ACER3 alterations. These include enzyme blockers in the event of overexpression and chemical chaperones in case of genetic deficiencies. Our results also alert about the importance of performing NC inhibitors screening programs in cell free systems and preferably, intact cells, since the use of rhNC affords results which are not recapitulated over the endogenous enzyme.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work has been partly funded by the Spanish Ministry of Economy, Industry and Competitiveness (grant CTQ2017-85378-R) and Fundación BBVA (grant 35_2018). We thank Dr. Gemma Triola for critically reading the manuscript, and Alexandre Garcia, Pedro Rayo, Neus Roca and Eva Dalmau for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113296.

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