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Development of new inhibitors for N-acylethanolamine-hydrolyzing acid amidase as promising tool against bladder cancer

Riccardo Vago [a,b], Arianna Bettiga [a], Andrea Salonia [a,b], Pierangela Ciuffreda [c], Roberta Ottria [c,*]

[a] Urological Research Institute, Division of Experimental Oncology, IRCCS San Raffaele Hospital, Via Olgettina 60, Milan, Italy
[b] Università Vita-Salute San Raffaele, Via Olgettina 60, Milano, Italy
[c] Dipartimento di Scienze Biomediche e Cliniche "Luigi Sacco", Via G.B. Grassi 74, Università degli Studi di Milano, Italy

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ABSTRACT

The endocannabinoid system is a signaling system involved in a wide range of biological effects. Literature strongly suggests the endocannabinoid system role in the pathogenesis of cancer and that its pharmacological activation produces therapeutic benefits. Last research promotes the endocannabinoid system modulation by inhibition of endocannabinoids hydrolytic enzymes instead of direct activation of endocannabinoid receptors to avoid detrimental effects on cognition and motor control. Here we report the identification of N-acylethanolamine-hydrolyzing acid amidase (NAAA) inhibitors able to reduce cell proliferation and migration and cause cell death on different bladder cancer cell lines. These molecules were designed, synthesized and characterized and active compounds were selected by a fluorescence high-throughput screening method set-up on human recombinant NAAA that also allows to characterize the mechanism of inhibition. Together our results suggest an important role for NAAA in cell migration and in inducing tumor cell death promoting this enzyme as pharmacological target against bladder cancer.

1. Introduction

Endocannabinoids (EC) and N-acylethanolamides (NAEs) are lipid mediators of an endogenous signaling system, the endocannabinoid system (ECS), transiently active, involved in a wide range of biological effects. The ECS includes EC, NAEs, their specific receptors and biosynthetic and metabolic pathways.[1] EC and NAEs biological effects, indeed, depend on their life span in extracellular space limited by receptor internalization upon interaction and enzymatic degradation. Arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) are the earliest described lipid derivatives from the EC family that also comprise other arachidonic acid derivatives. AEA also belongs to NAEs, which comprises non-endocannabinoids as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), while 2-AG does not. The literature strongly suggests a role for the ECS in the pathogenesis of cancer promoting ECS as promising therapeutic target for cancer treatment.[2] Latest studies propose the involvement of EC and NAEs in maintaining balance in cell proliferation demonstrating that targeting ECS can affect cancer growth.[3] ECS has been demonstrated to act by several different cellular mechanisms including inhibition of cell proliferation, migration and progression, inhibition of angiogenesis and promotion of apoptosis and/or cell cycle arrest. Although numerous evidences that pharmacological activation of ECS by cannabinoid receptors (CB) can elicit therapeutically beneficial effects, the associated detrimental effects of CB agonists on cognition and motor control limit their extensive use as pharmaceuticals. Accordingly, pharmacological blockade of the EC hydrolytic enzymes such as fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) has emerged as a potentially attractive strategy to increase EC signaling and retain advantages of CB activation avoiding undesirable side effects. At the same time, the inhibition of EC and NAEs hydrolyzing activity will reduce production of eicosanoids and free fatty acids, important pro-inflammatory mediators.

In last few years, the availability of selective inhibitors for EC hydrolytic enzymes has allowed the identification of EC role in some pathologies, and provided preclinical data supporting potential applications for this class of compounds.[4] Moreover, the overexpression of FAAH or MAGL has been demonstrated in aggressive cancers.[5] More recently, N-acylethanolamine-hydrolyzing acid amidase (NAAA),[6] a new NAEs degrading enzyme, not related to FAAH,

* Corresponding author at: Università degli Studi di Milano-Dipartimento di Scienze Biomediche e Cliniche "Luigi Sacco", Via G.B. Grassi 74, 20157 Milano, Italy.
E-mail addresses: vago.riccardo@hsr.it (R. Vago), bettiga.arianna@hsr.it (A. Bettiga), andrea.salonia@hsr.it (A. Salonia), pierangela.ciuffreda@unimi.it (P. Ciuffreda), roberta.ottria@guest.unimi.it (R. Ottria).

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belonging to the choloylglycine hydrolase family, has been cloned and characterized. NAAA shows a pH optimum at 4.5–5 and is almost inactive at alkaline or neutral pH; it has been shown to be highly expressed in macrophages and localized in lysosomes of various rat tissues including lung, spleen, intestine and brain. Although FAAH and NAAA can hydrolyze various NAEs, the substrates preferred are different; indeed, NAAA hydrolyses preferentially palmitoylethanolamide (PEA) and monounsaturated NAEs like oleoylethanolamide (OEA). In the last years, some selective NAAA inhibitors have been developed as anti-inflammatory[7] and/or analgesic[8] compounds promoting this enzyme as a promising pharmacological target in this area. Bladder cancer is one of the most common and expensive malignancies in developed countries due to the need for continuous monitoring and recurrence treatment. Its treatment remains a challenge despite significant improvements in preventing disease progression, because of its high rate of recurrence and potential progression, regardless of treatment with surgery, chemotherapy, or immunotherapy.[9] Alternative and/or complementary strategies to state-of-art therapies are urgently needed to limit the recurrence and improve survival. Although the ECS has been extensively studied in the field of cancer,[10] to date there is only one study regarding bladder cancer and the ECS.[11] Gasperi and co-workers demonstrated that functional CB-receptors are expressed in bladder cancer cells and that their stimulation by an inverse agonist exerts anti-proliferative effects. Given the presence of ECS components in bladder cancer cells and their involvement in cancer cell proliferation, our aim was to explore the therapeutic utility of NAAA inhibitors in bladder cancer. In the present work, we report the synthesis and the complete characterization of eight new compounds with the evaluation of their inhibitory activity toward human NAAA. Moreover, we show that the most effective NAAA inhibitors are able to significantly inhibit migration and drastically reduce viability and proliferation of bladder cancer cells resembling different disease stages and proliferation rate.

2. Results and discussion

2.1. Design and synthesis of new NAAA inhibitors

To date, some different classes of compounds have been reported to selectively inhibit NAAA. Vandevoorde and coworkers firstly proposed retroamides, esters and retroesters of palmitic acid as NAAA inhibitors.[12] The most potent inhibitor to date described from the class of palmitic acid-retroamides are N-pentadecylbenzylamide (**a**) and N-pentadecylhydroxypropylamide (**b**) shown in Fig. 1.[13] Starting from data reported in literature on NAAA palmitic acid-retroamide inhibitors,[14] we decided to synthesize a small library of retroamide derivatives of palmitamine to explore how structural modification such as the presence of one or more heteroatoms (hydrophilic interactions promoters), and their stereochemical position, or double aromatic ring could influence inhibitory ability. In detail we synthesized benzoic (**1**), hydroxypropylic (**2**), salicylic (**3**), R-mandelic (**4**) and S-mandelic (**5**), pyridinecarboxylic (**6**), 2,5-diidroxybenzoic (**7**) and 2-naphthoic (**8**) acid derivatives of hexadecylamine (Fig. 1). All compounds were synthesized by a one-step coupling reaction of the appropriate carboxylic acid with hexadecylamine, according to the procedure previously described for the synthesis of N-acylethanolamines[15] with yields that range from 45% to 85%.

2.2. Selection of inhibitors by a high-throughput screening fluorimetric method

It has been recently demonstrated that the non-fluorescent N-(4-methyl coumarin)palmitamide (PAMCA) can be a useful probe for NAAA enzymatic activity because it is hydrolyzed by NAAA, producing the fluorescent 7-amino-4-methylcoumarin, with a binding affinity similar to or slightly greater than that for PEA.[16] A fluorescent-based assay for NAAA activity was selected to assess inhibitory activity of new synthesized compounds. This kind of methods are simple, sensitive, time saving, with rapid read out and cheap, all characteristics suitable for high throughput screening of small or large library of new compounds. According to this finding PAMCA was freshly synthesized from palmitic acid and 7-methylamino coumarine and, after purification and complete characterization, it was used to set-up the fluorescent-based assay on human recombinant NAAA (hrNAAA, purity >95%). Enzymatic reaction conditions were optimized for the screening of new designed inhibitors using compound **1** and **2** selected as models of aromatic and linear palmitamine derivatives and dimethylsolfoxide (DMSO) as blank. Different quantities of the enzyme and concentrations of PAMCA have been assessed and finally 25 ng of hrNAAA and 10 μM PAMCA have been selected as the amount with better fluorescence production in reaction time. In a typical analysis, various concentrations of test compound dissolved in DMSO or DMSO alone were added to the reaction buffer containing hrNAAA. After incubation with the inhibitor, PAMCA, was added and the assay ran for 5 h. The kinetic curves obtained in optimized reaction condition with compound **1** at four concentrations (5, 10, 50, 100 μM final concentrations per well) showed that hydrolysis rate of PAMCA by NAAA was linear for all 5 h (Fig. 2). To obtain a useful tool for high throughput screening we decided to stop the assay at 2.5 h. At first, we selected three scalar logarithmic concentrations to test the activity of our potential inhibitors of NAAA. Compounds at 1, 10 and 100 μM final concentrations were incubated together with NAAA and fluorescence intensity was monitored after addition of the substrate PAMCA. Residual enzymatic activity was calculated from slops of kinetic curves and expressed as percentage. From this preliminary screening, compounds **3**–**7** showed a concentration-dependent inhibitory activity while only 2-naphthoic acid derivative **8** did not show any inhibition toward NAAA. Consequently, compounds **1**–**7** were selected for further investigation.

2.3. Characterization of active compounds and structure-activity relationship consideration

Five points assay (10-25-50-75 and 100 μM final concentrations) was performed with compounds **1**–**7** to obtain inhibition curves and IC_{50} values. From the slop of kinetic curves, residual enzymatic activity was calculated and IC_{50} values were obtained by non-linear regression analysis of Log[concentration]/inhibition curves. IC_{50} values found in our experimental conditions are listed in Fig. 1 along with chemical structures of synthesized compounds. Compounds **1** and **2** showed IC_{50} values of 45 ± 1.6 and 34 ± 1.2 μM respectively. Interestingly compounds **3** and **4** exert three and two fold major inhibitory activity toward NAAA with IC_{50} values of 11 ± 1.0 and 20 ± 0.9 μM respectively, while inhibitory activity of compound **7** is similar to compound **2** (IC_{50} value of 38 ± 1.0 μM). Notably, compound **4**, the R-mandelic acid derivative, displayed three fold higher activity than its enantiomer **5** (IC_{50} 78 ± 2.8 μM). Finally compound **6** was the less active in hrNAAA inhibition, displaying an IC_{50} of 98 ± 1.0 μM. Starting from these results compounds **3**, **4** and **7**, along with compound **2**, the only linear palmitamine derivative, were selected to examine their inhibition mechanism. The effects of different concentrations of PAMCA with hrNAAA in the presence or absence of inhibitors (100 μM) were analyzed. Results reported in the Lineweaver-Burk plot, shown in Fig. 3, demonstrate a competitive inhibition for all new compounds tested.

We also examined the reversibility of the inhibition incubating NAAA with selected compounds for 20 min. After incubation, solu-

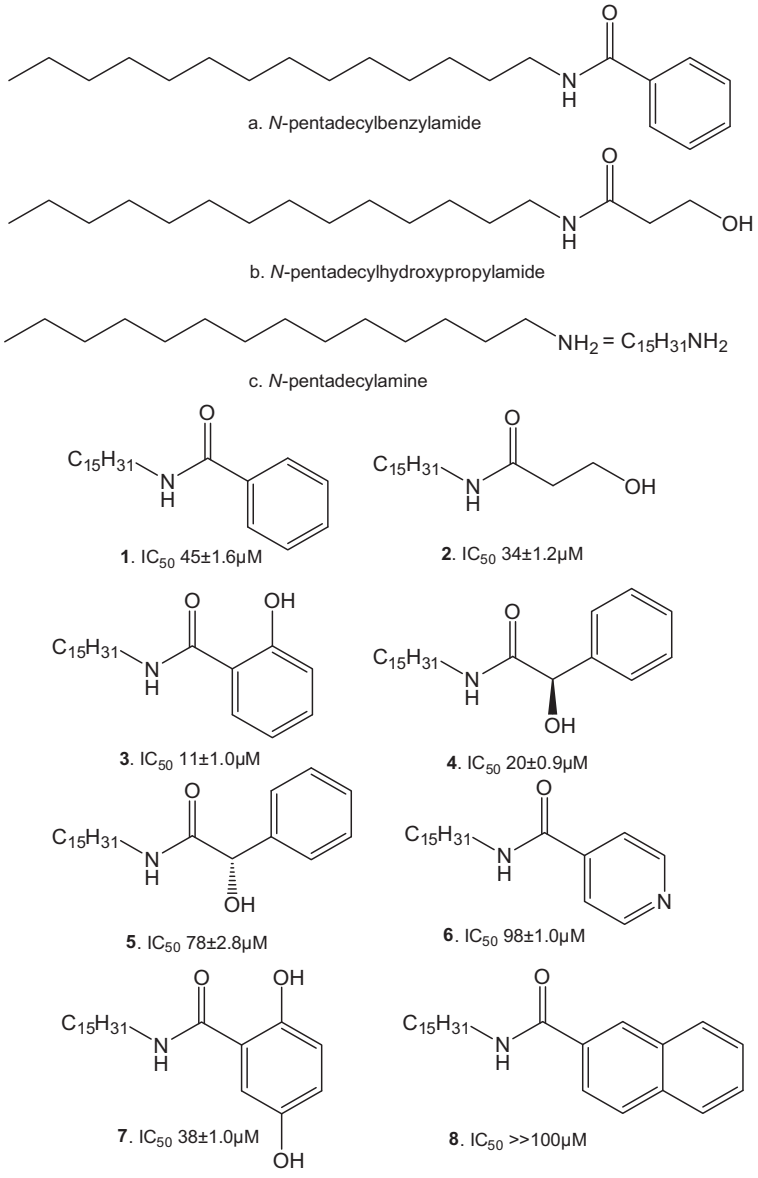

Fig. 1. Chemical structures and IC_{50} (μM) values of synthesized compounds in direct inhibition of hrNAAA.

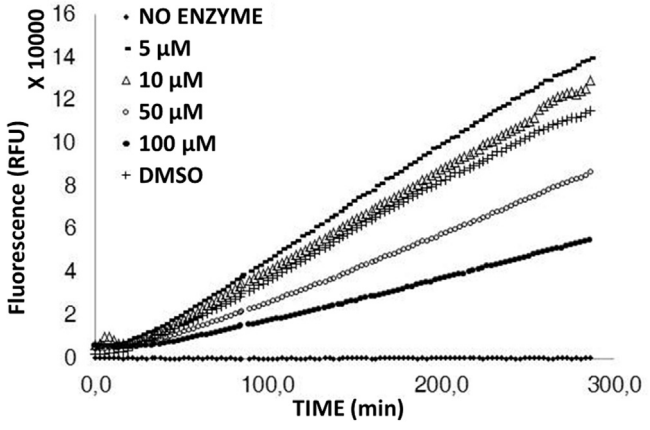

Fig. 2. Kinetic curves of inhibition of hrNAAA by compound **1** at four concentrations: 5-10-50-100 μM (RFU = Relative fluorescence Emission).

tions were diluted twenty fold and new amounts of inhibitors were added; then reaction was started by PAMCA addition. Results show that the inhibitory activity of compounds **2** and **4** was determined mostly by the second concentration added rather than by its initial concentration, suggesting the reversibility of the inhibition (Fig. 4). Unfortunately, this experiment has not yielded reliable results for the samples **3** and **7** because of the intrinsic fluorescence of the molecules. Taken together these results suggest that the presence of one aromatic ring maintain the inhibitory activity of the molecule while a double ring results in a loss of activity. Moreover, the presence of one or two hydroxyl groups on the ring, promoter of hydrophilic interactions with the enzyme, ameliorates the inhibition power.

2.4. NAAA inhibitors affect bladder cancer cells viability

Since ECS has been demonstrated to play a critical role in tumors[17] and enzymes involved in EC metabolism have been found more expressed in cancer,[18,19] we tested the effect of our NAAA

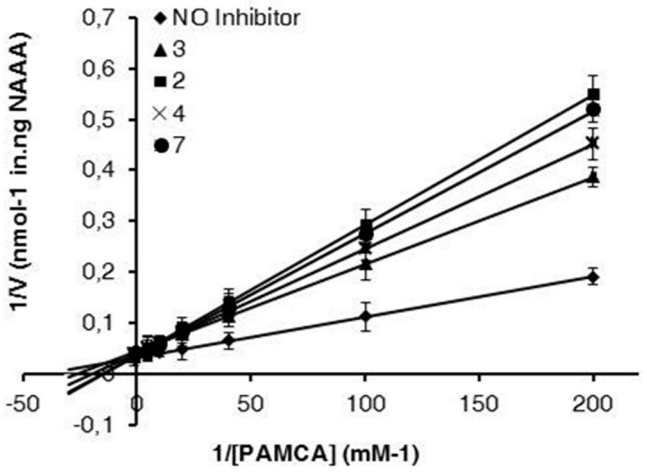

Fig. 3. Lineweaver-Burk plot of the inhibition of hrNAAA by compounds **2**, **3**, **4** and **7**.

inhibitors on human bladder cancer cell lines. To address tumor heterogeneity, we used different cell lines resembling various stages of the disease, named RT4, RT112, 5637 and HT-1376. They differ in size, proliferation rate and motility, making a sample panel for the analysis. We assayed cell viability by incubating cells with serial logarithmic dilutions of NAAA inhibitors ranking from 100 to 0.1 μM for 72 h and then a MTT assay was performed (Table 1). As for the cell-free assay, the inhibitors showed different activity and compound **3** confirmed to be the most effective in reducing cell proliferation and causing cell death with an IC_{50} much lower than the others in all cell lines analyzed. Compound **1** was the less active displaying a definable IC_{50} only in 5637 cells experiment. Compound **2** was effective only on RT112 and 5637 cells proliferation displaying a quite high IC_{50} value. Compounds **4** and **5** corroborated previous data, displaying increased activity compared to compound **2**. Notably, compound **7** resulted highly active, in a very similar extent to compound **3**. HT-1376 cells were originally derived from a transitional cell carcinoma of grade III, the highest in the panel; they bear more invasive and metastatic properties than stage II 5637[20] and are sensitive only to compounds **3** and **7**. Nevertheless, they are little (**3**) or not active (**7**) on grade I RT4, making them suitable for the treatment of more aggressive bladder tumors. The compound **4** is, instead, the best performers on RT4 cells, but is inactive on HT-1376.

2.5. Effects of NAAA inhibitors on the bladder cancer cell migration

Tumor cell migration is an important step for the spread of cancer and ECS has been proven to be involved in this process.[17] Since RT112 cells are characterized by rapid migration, we investigated the role of NAAA inhibitors on cell migration in a classical wound healing assay. Sub-confluent cells were scratched and incubated with compounds **3** and **7**, the overall best inhibitors of NAAA activity and cell viability, or with compound **2** as the only linear palmitamine derivative. After 24 h treatment, wound closure was calculated. As shown in Fig. 5, untreated RT112 cells migrated very fast and almost completely covered the scratch after 24 h. Compound **2** exerted a very modest effect, while compounds **3** and **7** significantly reduced cell migration (50% and 80% respectively). These results suggest an important role for NAAA in the cell migration and its inhibition as a potential approach to reduce cell migration and thus tumor dissemination. The overall data demonstrate that a new class of NAAA inhibitors can be effective in inducing tumor cell death, paving the way for a future development of these compounds for cancer treatment.

2.6. Effects of inhibitors on endocannabinoids levels in bladder cancer cells

To assess how the treatment of bladder cancer cells with synthesized inhibitor affected the EC and NAEs levels, the following EC and NAEs N-palmitoylethanolamide (PEA, C16:0), N-stearoylethanolamide (SEA, C18:0), N-oleoylethanolamide (OEA, C18:1), anandamide (N-arachidonoylethanolamide, AEA, C20:4), N-linoleoylethanolamide (LEA, C18:2), N-α-linolenoylethanolamide (LNEA, C18:3) and N-eicosapentaenoylethanolamide (EPEA, C20:5) have been quantified by HPLC-MS/MS analysis. Cells and supernatants were extracted and purified as described in the experimental part and quantification results, expressed in ng/ml, are reported in Fig. 6. Fig. 6 lacks of EPEA because it was undetectable or its levels were under the limit of quantification of the method in all samples. At our experimental conditions, 24 h of treatment with 40 μM inhibitor on RT112 cell line, all assessed inhibitors affect concentrations of N-acylethanolamides of saturated or monounsaturated acids while polyunsaturated acid derivatives concentrations remained unvaried. In detail all com-

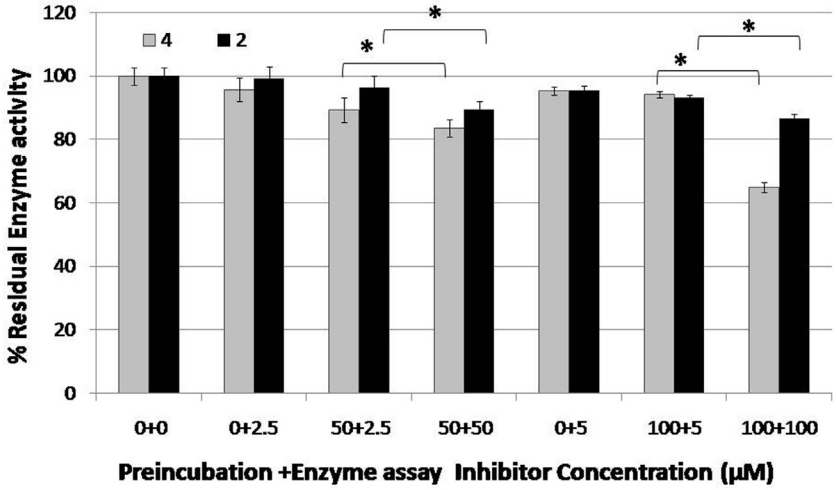

Fig. 4. Reversibility of the inhibition of hrNAAA by compounds **2**, and **4**. *$p < 0.05$.

Table 1
Cell viability of bladder cancer cell lines treated with NAAA inhibitors. RT4, RT112, 5637 and HT-1376 cells were incubated with compounds **1–7** and after 72 h MTT assay was performed. IC_{50} is indicated ± standard deviations. NM = Not Measurable.

	RT4	RT112	5637	HT-1376
1 (μM)	N M	N M	45.00 ± 22.32	N M
2 (μM)	N M	58.33 ± 23.63	40.00 ± 10.95	N M
3 (μM)	63.33 ± 20.82	5.75 ± 1.63	11.25 ± 5.97	10.25 ± 3.69
4 (μM)	3.50 ± 2.18	28.33 ± 2.89	25.00 ± 9.13	N M
5 (μM)	>100 μM	25.00 ± 0.00	18.75 ± 8.54	N M
6 (μM)	41.67 ± 5.77	76.25 ± 7.50	51.25 ± 16.52	N M
7 (μM)	N M	7.00 ± 0.96	7.25 ± 2.22	9.50 ± 1.00

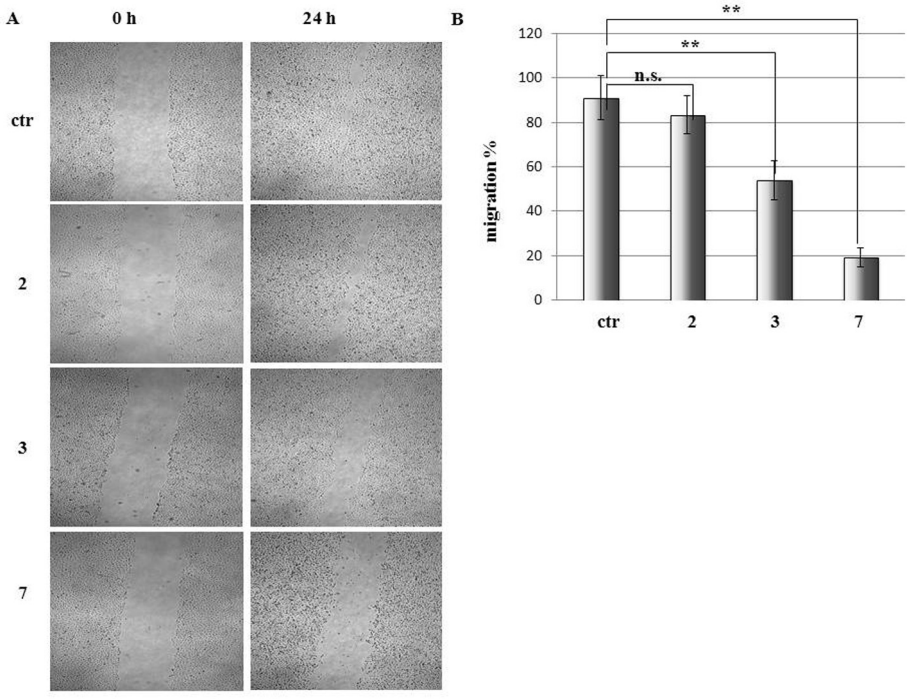

Fig. 5. Effects of NAAA inhibitors on the bladder cancer cell migration. The migration capacity of RT112 bladder cancer cells were tested in the presence of NAAA inhibitors **2**, **3** and **7** in a wound healing assay. Panels (A) shows representative pictures of RT112 cells at time 0 and after 24 h treatment. The migration was determined by the rate of cells filling the scratched area and the result represented as mean ± standard deviation (B). Ctr.: negative control without inhibitors. $^{**}p$ value < 0.01; n.s.: not significant.

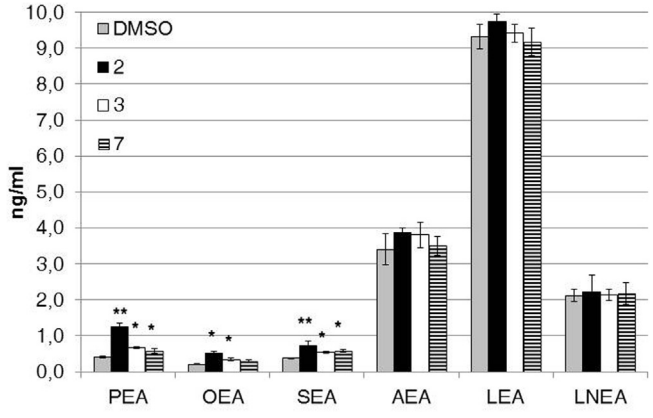

Fig. 6. Quantifications of EC and NAEs by HPLC-MS/MS analysis. EC and NAEs levels in RT112 and 5637 bladder cancer cells. $^{*}p < 0.05$, $^{**}p < 0.01$.

pound caused a statistically significant increment of PEA in RT112 cell line. OEA was incremented by the three molecules assessed, but only variation linked to compound **2** and **3** treatment were statistically significant. Finally, SEA concentration was incremented in RT112 by all assessed compounds. Together these results demonstrate the selective inhibition of NAAA in intact bladder cancer cells since they increase the levels of PEA, SEA and OEA without affecting concentrations of the other EC and NAEs AEA, LEA and LNEA.

2.7. Statistics

All experiments on hrNAAA and on cell lines have been performed at least three times in triplicate and results are expressed as mean of obtained results ± standard deviation. Significance of experiments was assessed by Student's *T*-test.

3. Conclusions

Here we report the design and synthesis of a small library of retroamide derivatives of palmitamine with the aim to identify new NAAA inhibitors. Moreover, we describe the set-up of a fluorescent base method to assess enzymatic activity, fast, sensitive and cheap, that allow the high-throughput screening of newly synthesized compounds on human recombinant NAAA and the characterization of the inhibition mechanism of active molecules. In addition, we demonstrate that three of new compounds are active on hrNAAA at low concentration and are able to reduce cell proliferation and

cause cell death on different bladder cancer cell lines. Obtained results from cell migration experiments demonstrate that compounds **3** and **7** significantly reduce cell migration while inhibitor **2** shows a very low effect suggesting an important role for NAAA in the cell migration. The overall data demonstrate that a new class of NAAA inhibitors can induce tumor cell death and reduce cell migration promoting NAAA as new target in bladder cancer management and paving the way for a future development of these compounds for cancer treatment. Results from in vitro experiments on hrNAAA allow considerations on structure activity relationship and lay the bases to optimize additional molecules as NAAA inhibitors. Finally, the increase of PEA, SEA and OEA without modifications in the concentrations of the other EC and NAEs AEA, LEA and LNEA is an additional evidence of the selective inhibition of NAAA in intact bladder cancer cells. Moreover, these results demonstrate that effects on survival and migration of bladder cancer cells are due to NAAA inhibition. Comparing our data with the to date knowledge on the involvement of ECS in cancer management we can hypothesize that PEA, OEA and SEA increase could affect bladder cancer by several mechanisms as apoptosis promotion, intensification of the apoptotic response induced by AEA, or angiogenesis modulation. Moreover OEA has been recognized as acid ceramidase (AC) inhibitor[21] concurring to sensitize prostate bladder cells to EC–induced apoptosis. Further experiments are required and will be devoted to confirm the hypothesized mechanism of action of NAAA inhibitors.

4. Materials and methods

4.1. Chemistry

Column chromatography was performed on Silica Gel 60 (70–230 mesh) using the specified eluents. The progress of the reactions was monitored by analytical thin-layer chromatography (TLC) on pre-coated glass plates (silica gel 60 F254-plate-Merck, Darmstadt, Germany) and the products were visualized by UV light. Purity of all compounds (>99%) was verified by thin layer chromatography and NMR measurements. Elemental analyses were obtained for all intermediates and are within ±0.4% of theoretical values. The chemicals and solvents were obtained from Sigma–Aldrich and used without further purification. Melting points were determined with a Stuart Scientific SMP3 melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter (sodium D line at 25 °C). ^{1}H NMR spectra were recorded in $CDCl_3$ (isotopic enrichment 99.95%) or pyridine-d5 (isotopic enrichment 99.98%) solutions at 300 K using a Bruker AVANCE 500 instrument (500.13 MHz for ^{1}H) using 5 mm inverse detection broadband probes and deuterium lock. Chemical shifts (δ) are given as parts per million relative to the residual solvent peak (7.26 ppm for ^{1}H) and coupling constants (J) are in Hertz. The experimental error in the measured ^{1}H-^{1}H coupling constants is ±0.5 Hz. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, and bs, broad peak. For two-dimensional experiments, Bruker microprograms using gradient selection (gs) were applied.

All compounds were synthesized by a one-step coupling reaction. Briefly, in a general procedure the appropriate acid (1 eq), and (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluoro phosphate (COMU, 1 eq) were dissolved in anhydrous CH_2Cl_2/CH_3CN (3/1) at room temperature under a nitrogen atmosphere. After 10 min diisopropylethylamine (2 eq) was added and the resulting orange-red solution was stirred for 30 min. Hexadecylamine (1 eq) in CH_3CN was then injected into the reaction mixture and vigorous stirring at room temperature was continued until TLC confirmed the completion of the reaction (1–6 h). The reaction mixture was diluted with CH_2Cl_2 and washed with 5% HCl, saturated $NaHCO_3$ and brine. The organic layers were collected, dried over anhydrous Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure and crude purified by flash chromatography to give white solids and reaction yields range from 45% to 85%.

4.1.1. N-Hexadecylbenzamide (*1*)

The compound **1** was prepared from benzoic acid (50 mg, 0.41 mmol) as white solid 122 mg (0.35 mmol, 85%) Rf 0.42 (Hexane/Acetone 85:15). Mp 83-85 °C. ^{1}H NMR: δ 0.90 (3H, t, J = 6.9, 16′-CH_3), 1.22–1.40 (24 H, m, 4′-15′-CH_2), 1.63 (2H, tt, J = 7.0, 7.0, 3′-CH_2), 3.47 (2H, t, J = 7.0, 2′-CH_2), 6.18 (1H, bs, NH), 7.45 (2H, dd, J = 7.0, 7.0, *m*-Ph H), 7.51 (1H, t, J = 7.0, *p*-Ph H), 7.78 (2H, d, J = 7.0, *o*-Ph H). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7(15′-CH_3), 27.0, 29.3, 29.4, 29.6, 29.7, 29.8, 31.9 (14 × CH_2), 40.1 (2′-CH_2), 126.9, 128.5, 131.1, 134.9 (aromatic-*C*), 167.5 (*C*=*O*). Physical and spectroscopic data were in accordance with those reported.[22]

4.1.2. N-(3-Hydroxypropionyl)-hexadecananamide (*2*)

The compound **2** was prepared from 3-hydroxypropanoic acid (48 mg, 0.53 mmol, d 1.08 g/mL, 34 μL) as white solid 69 mg (0.22 mmol, 42%). Rf 0.38 (Hexane/Acetone 80:20). Mp 85–87 °C. ^{1}H NMR ($CDCl_3$): δ 0.91 (3H, t, J = 6.9, 16′-CH_3), 1.25–1.36 (24 H, m, 4′-15′ CH_2), 1.55 (2H, tt, J = 7.0, 7.0, 3′-CH_2), 2.54 (2H, t, J = 5.4, 1-CH_2), 3.31 (2H, t, J = 7.0, 2′-CH_2), 3.93 (2H, t, J = 5.4, 2-CH_2), 6.30 (1H, bs, NH). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 26.9, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 31.9 (14 × CH_2), 37.7 (2-CH_2), 39.6 (2′-CH_2), 59.0 (1-CH_2), 173.3 (*C*=*O*). Anal. Calcd. for $C_{19}H_{39}NO_2$ (313,30): C, 72.79; H, 12.54; N, 4.47. Found: C, 72.77; H, 12.53; N, 4.48.

4.1.3. N-Hexadecyl-2-hydroxybenzamide (*3*)

The compound **3** was prepared from 2-hydroxybenzoic acid (57 mg, 0.41 mmol) as white solid 109 mg (0.30 mmol, 73%). Rf 0.48 (Hexane/Acetone 80:20). Mp 104–106 °C. ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, J = 6.5, 16′-CH3), 1.25–1.34 (24 H, m, 4′-15′ CH_2), 1.65 (2H, tt, J = 7.0, 7.0, 3′-CH_2), 3.47 (2H, t, J = 7.0, 2′-CH_2), 6.87 (1H, ddd, J = 1.4, 7.6, 7.7, 5-H), 7.01 (1H, dd, J = 1.4, 7.7, 3-H), 7.36 (1H, dd, J = 1.4, 7.6, 6-H), 7.41 (1H, ddd, J = 1.4, 7.6, 7.7, 4-H), 8.26 (1H, bs, NH). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 27.0, 29.3, 29.4, 29.6, 29.7, 29.8, 31.9 (14 × CH_2), 39.7 (2′-CH_2), 114.4, 118.5, 118.7, 125.1, 134.1 (aromatic-*C*), 161.2 (2-C), 169.9 (*C*=*O*). Physical and spectroscopic data were in accordance with those reported.[23]

4.1.4. N-Hexadecyl-(R)-2-Hydroxy-2-phenylaceticamide (*4*)

The compound **4** was prepared from *R*-mandelic acid (63 mg, 0.41 mmol) as white solid 106 mg (0.28 mmol, 68%). Rf 0.56 (Hexane/Acetone 80:20). Mp 90–92 °C. $[\alpha]_D^{20}$-5.0 (c 1, $CDCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, J = 6.7, 16′-CH_3), 1.22–1.34 (24 H, m, 4′-15′ CH_2), 1.48 (2H, tt, J = 7.0, 7.0, 3′-CH_2), 2.65 (1H, bs, OH), 3.27 (2H, t, J = 7.0, 2′-CH_2), 5.04 (1H, s, 1-H), 6.07 (1H, bs, NH), 7.36–7.44 (5H, m, aromatic). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 26.7, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 31.9 (14 × CH_2), 39.7 (2′-CH_2), 74.1 (1-C), 126.9, 128.7, 128.9, 139.6 (aromatic-*C*), 172.0 (*C*=*O*). Anal. Calcd for $C_{24}H_{41}NO_2$ (375,31): C, 76,75; H, 11.00; N, 3.73. Found: C, 76.74; H, 10.99; N, 3.75.

4.1.5. N-Hexadecyl-(S)-2-hydroxy-2-phenylaceticamide (*5*)

The compound **5** was prepared from *S*-mandelic (63 mg, 0.41 mmol) as white solid 114 mg (0.30 mmol, 73%). Rf 0.56 (Hexane/Acetone 80:20). Mp 89–92 °C. $[\alpha]_D^{20}$ + 5.1 (c 1, $CDCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, J = 6.7, 16′-CH_3), 1.22–1.34 (24 H, m, 4′-15′ CH_2), 1.48 (2H, tt, J = 7.0, 7.0, 3′-CH_2), 2.66 (1H, bs, OH), 3.27 (2H, t, J = 7.0, 2′-CH_2), 5.04 (1H, s, 2-H), 6.07 (1H, bs, NH), 7.36–7.44

(5H, m, aromatic). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 26.7, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9 (14 × CH_2), 39.7 (2′-CH_2), 74.1 (1-C), 126.9, 128.7, 128.9, 139.6 (aromatic-*C*), 172.0 (*C*=*O*). Anal. Calcd. for $C_{24}H_{41}NO_2$ (375,31): C, 76.75; H, 11.00; N, 3.73. Found: C, 76.77; H, 10.98; N, 3.71.

4.1.6. N-Hexadecyl-4-pyridincarboxyamide (**6**)

The compound **6** was prepared from 4-pyridinecarboxylic acid (51 mg, 0.41 mmol) as white solid 124 mg (0.36 mmol, 88%). Rf 0.64 (Hexane/Acetone 75:25). Mp 93–94 °C. ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, *J* = 6.7, 16′-CH_3), 1.24–1.38 (24 H, m, 4′-15′ CH_2), 1.66 (2H, tt, *J* = 7.0, 7.0, 3′-CH_2), 3.49 (2H, t, *J* = 7.0, 2′-CH_2), 6.79 (1H, bs, NH), 7.85 (2H, d, *J* = 5.3 Hz, 3 and 5-H), 8.80 (2H, d, *J* = 5.3 Hz, 2 and 6-H). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 27.0, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 31.9 (14 × CH_2), 40.5 (2′-CH_2), 122.0, 144.0, 148.4, (aromatic-*C*), 164.6 (*C*=*O*). Physical and spectroscopic data were in accordance with those reported.[24]

4.1.7. N-Hexadecyl-2,5-dihydroxybenzamide (**7**)

The compound **7** was prepared from 2,5-dihydroxybenzoic acid (64 mg, 0.42 mmol) as white solid 75 mg (0.20 mmol, 48%). Rf 0.70 (Hexane/Acetone 70:30). Mp 101–103 °C. ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, *J* = 6.7, 16′-CH_3), 1.23–1.38 (24 H, m, 4′-15′ CH_2), 1.62 (2H, tt, *J* = 7.0, 7.0, 3′-CH_2), 3.42 (2H, t, *J* = 7.0, 2′-CH_2), 6.85 (1H, bd, *J* = 8.0, 4-H), 6.91 (1H, bs, 6-H), 6.93 (1H, bd, *J* = 8.0, 3-H), 8.24 (1H, bs, NH). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 27.0, 29.3, 29.4, 29.6, 29.7, 29.8, 31.9 (14 × CH_2), 39.7 (2′-CH_2), 111.3, 118.9, 122.1 (aromatic-*C*), 148.3 (2-C), 154.4 (4-C), 169.5 (*C*=*O*). Physical and spectroscopic data were in accordance with those reported.[25]

4.1.8. N-Hexadecylnaphthalene-2-carboxamide (**8**)

The compound **8** was prepared from 2-naphthoic acid (72 mg, 0.42) as white solid 139 mg (0.35 mmol, 83%). Rf 0.58 (Hexane/Acetone 70:30). Mp 107–109 °C. ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, *J* = 6.7, 16′-CH_3), 1.27–1.46 (24 H, m, 4′-15′ CH_2), 1.69 (2H, tt, *J* = 7.0, 7.0, 3′-CH_2), 3.54 (2H, t, *J* = 7.0, 2′-CH_2), 6. 29 (1H, bs, NH), 7.54–7.60 (2H, m, 6- and 7-H), 7.85 (1H, dd, *J* = 1.8, 8.5 Hz, 3-H); 7.89–7.95 (3H, m, 4-, 5-and 8-H), 8.30 (1H, d, *J* = 1.8 Hz, 1-H). ^{13}C NMR: δ 14.1 (16′-CH_3), 22.7 (15′-CH_3), 27.1, 29.4, 29.5, 29.6, 29.7, 31.9 (14 × CH_2), 40.3 (2′-CH_2), 123.6, 126.7, 127.2, 127.5, 127.7, 128.4, 128.9, 132.1, 132.7, 134.7 (aromatic-*C*), 167.5 (*C*=*O*). Anal. Calcd. for $C_{27}H_{41}NO$ (395,32): C, 81.97; H, 10.45; N, 3.54. Found: C, 81.98; H, 10.46; N, 3.52.

4.2. Selection of inhibitors by an high-throughput screening fluorimetric method

The fluorogenic probe was synthesized starting from palmitic acid and 7-methyl-4-amino coumarine, by a coupling reaction and in presence of COMU and DIPEA in dry condition as described for compounds **1**–**7**. Crude was purified by flash chromatography (Hexane/Acetone 80:20) to give product as white solid (95 mg, 0.23 mmol) and reaction yield of 56%. NMR analyses were in agreement with that described in literature[16] confirming structure and purity grade of the product. The florescence assay procedure used to optimize the method to evaluate NAAA activity was very similar to that used by West and coworkers.[16] We decided to performed in vitro experiments only in presence of hrNAAA, instead of a total protein extract from cells over-expressing NAAA, because PAMCA is not a substrate specific for this enzyme and the presence of FAAH or other hydrolases could influence florescence production. To optimize the experiment condition different amounts of hrNAAA (R&D System, Cod 4494-AH, purity >95%) and concentration of PAMCA were tested. In a 96 well black plate to each well, containing 180 μl of reaction buffer (sodium acetate buffer 100 mM (pH 4.75), 3 mM DTT and 0.1% Triton X-100), the correct amount of hrNAAA (5-15-25-50 ng) and 20 μl of a solution of PAMCA in DMSO (to obtain final concentration of PAMCA of 1-5-10,100 μM) were added. The reaction was run for 5 h in a Perkin Elmer Victor 3 fluorimeter with fluorescence readings taken every 3 min at a wavelength of 460 nm applying an excitation wavelength of 360 nm. As described before 25 ng of hrNAAA and PAMCA 10 μM were selected as the amount with better fluorescence production in a good reaction time. Compound **1** was used to optimize screening experiments using conditions described above. 10 μl of DMSO solution of **1** (to reach 5-10-50-100 μM final concentrations per well) were added to hrNAAA in 180 μl of reaction buffer and after 30 min of incubation at room temperature 10 μl of PAMCA in DMSO (to reach 10 μM final concentration) were added. The first screening and the selection of newly synthesized compounds were performed in the same conditions at three concentration levels 1-10-100 μM in a run of 2.5 h. Results were plotted as time *vs* measured fluorescence and residual enzymatic activity was calculated as percentage comparing the slope of kinetic curves of PAMCA hydrolysis in the presence or absence of inhibitors.

4.3. Characterization of active compounds and structure activity relationship consideration

Five points assay was performed with all compounds, excluding the inactive compound **8**, to obtain inhibition curves and IC_{50} values. As described before hrNAAA and compounds at 10-25-50-75 and 100 μM final concentrations were incubated for 30 min and, after PAMCA addition, reaction was monitored for 2.5 h. The enzyme activity was evaluate by fluorescence produced, and inhibition expressed as percentage of the residual enzyme activity. IC_{50} values were calculated by non-linear regression analysis of Log[inhibitor concentration]/enzyme activity curves using GraphPad Prism 5 applying standard slop curve. To explore the manner of inhibition (competitive or non-competitive), reaction of six different concentrations of PAMCA (5-10-25-50-100-200 μM final concentrations per well) with hrNAAA in the presence or absence of more active inhibitors **3**, **4**, **7** and the linear **2** (100 μM) were performed as described before. Analyzing data with GraphPad Prism 5, Michaelis-Menten curves gave V_{max} and K_M values and Lineweaver-Burk plot was built reporting 1/V *vs* 1/[PAMCA]. Reversibility experiments were performed, as described in literature,[21] incubating hrNAAA with selected compound at two different concentrations (50 and 100 μM final concentration × well) for 20 min. After incubation solutions were diluted twenty fold and new amount of inhibitors (2.5–50 μM final concentration to 50 μM initial concentration and 5–100 final concentration to 100 μM initial concentration) were added, then reaction was started by PAMCA addition. The fluorescence produced was evaluated and comparison of the enzyme activity displayed by the two couple of experiments allowed to define reversibility of the inhibition.

4.4. Cell culture and cell viability assay

RT4 (grade I), RT112 (II), 5637 (II) and HT-1376 (III) human bladder cancer cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.

To test the effect of NAAA inhibitors on cell viability, cells were plated in 96-well plates at a cell density of 5×10^3 cells/well and treated with serial logarithmic dilutions of NAAA inhibitors ranking from 100 to 0.1 μM for 72 h. Control cultures received the respective volume of DMSO. At the end of the exposure period, MTT assay was performed according to the manufacturer's recommendations (Sigma-Aldrich). Purple formazan product was re-sus-

pended in DMSO and measured in a spectrophotometric microplate reader at 570 nm wavelength. Cell viability was evaluated as the concentration inhibiting the 50% incorporation in untreated control cells and is expressed as IC_{50}.

4.5. Wound healing assay

Human bladder cancer cells were plated in 6-well culture dishes at a cell density of 5×10^5 cells/well to create a sub-confluent monolayer. A ~1 mm wide scratch was made across the cell layer using a sterile pipette tip. Debris were removed and the growth medium was replaced with a medium containing 40 nM NAAA inhibitors (DMSO only for controls). Phase-contrast images were acquired at time 0 and after 24 h incubation. Wound closure areas were measured with ImageJ software by subtracting the total amount of grayscale pixel counted in the cell-free area remaining after 24 h from the initial wound area. Data are reported as means ± standard deviations. Cell motility was assessed as percentage of cell covered area with respect to controls.

4.6. Effects of inhibitors on endocannabinoid levels in bladder cancer cells

After migration experiments cells were used to quantify: PEA, OEA, SEA, AEA, LEA, and LNEA. Quantification were performed on an ABSciex 5600 TripleTOF mass spectrometer (AB Sciex, Foster-City, CA, USA) coupled with a LC system composed by an Agilent 1290 Infinity autosampler and an Agilent 1200 Infinity pump (Agilent Technologies, Waldbronn, Germany) as described before.[26] Briefly, 1 ml of cells and supernatants taken together was spiked with a solution of d_4-SEA and d_4-LNEA, used as internal standard for quantification, and 800 µl of cold acetone were added for protein precipitation. After centrifugation, supernatants were extracted and purified by SPE extraction on OASIS HBL cartridges. Eluates were collected and maintained at −80 °C till the analyses. Quantifications were performed using Multiquant 1.2.1 software by AB Sciex. Data are reported as means ± standard deviations.

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A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.12.042.

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