Cytotoxicity of Endogenous Lipids N-acyl Dopamines and their Possible Metabolic Derivatives for Human Cancer Cell Lines of Different Histological Origin

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Abstract. *Background/Aim: Dopamine amides of long chain fatty acids are a family of endogenous mammalian lipids with an unknown function; they are anti-proliferative for the C6 glioblastoma cell line. To assess their possible anti-cancer activity we evaluated their cytotoxicity for a set of cancer cell lines. Materials and Methods: Anti-proliferative and cytotoxic actions of these substances were evaluated in HOS, IMR-32, MCF-7, Namalwa, K-562 and HEK 293 cell lines (18 h incubation time) using MTT and lactate dehydrogenase (LDH) tests, accordingly. Results: All N-acyl dopamines (NADA) induced cell death in all cell lines tested with a 50% lethal dose (LD_{50}) in the range of 0.5-80 μM, except for HEK-293. For HEK-293 only N-arachidonoyl epinephrine demonstrated an LD_{50} below 100 μM. Conclusion: According to the structure-activity relationship, N-acyl dopamines with an intact catechol group and a non-modified hydrophobic fatty acid residue are cytotoxic to cancer cell lines of various histological origins.*

N-acyl dopamines (NADA) are biogenic conjugates of dopamine with long chain fatty acids. Their endogenous nature was first proposed independently by Bezuglov *et al.* (1) and Pokorski and Matysiak (2). Subsequently, NADA were found in the nervous system of *Rattus norvegicus* and *Bos taurus* (0.1-6 pmol/g wet weight) (3, 4), in two species of the *Hydra* genus (5) and in human plasma (6). The residues of fatty acids of the naturally-occurring NADA encompass arachidonic, docosahexaenoic, oleic, palmitic and stearic acids. The targets of NADA are vanilloid receptor TRPV1 (activation, median effective concentration (EC_{50})=30-60 nM), cannabinoid receptor CB1 (activation, dissociation constant (K_d)=1-10 μM), calcium channels CaV3 (inhibition, half maximal inhibitory concentration (IC_{50})=0.3-1 μM), potassium channels TASK3 (inhibition, IC_{50} 2.4 μM), as well as several intracellular proteins. However, the exact function of these compounds *in vivo* is not clear (7).

The action of NADA on transformed or stressed cells deserves special attention as they protect the latter (8) and induce apoptosis in the former (9-11). More importantly, dopamine amides of arachidonic, oleic and palmitic acids inhibited growth of cancer stem cells enriched cultures (12). We suggested that such properties may be relevant to the NADA function *in vivo*. The aim of the present work was to investigate whether endogenous NADA and their tentative metabolites are cytotoxic for cancer cell lines of different histological origin.

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Materials and Methods

Human neuroblastoma IMR-32 and osteosarcoma HOS cell lines were purchased from the cell bank of the Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences, Saint-Petersburg, Russia). Human lymphoma K-562, leukemia Namalwa, carcinoma MCF-7 and HEK-293 cell lines were a kind gift of Dr. E. Smirnova (IBCh RAS, Russia). DMEM, RPMI 1640 and MEM cell medium, Hank's solution, HEPES, phosphate-buffered saline, streptomycin, penicillin, amphotericin B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), non-essential amino acids solution and trypsin-EDTA solution were from PanEco, Moscow, Russia. D-glucose, diaphorase, NAD^+, DL-lactic acid, Tris, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), bovine serum albumin, sucrose and sodium pyruvate were from Sigma-Aldrich, St. Louis, MO, USA. Fetal bovine serum was from PAA Laboratories, Pasching, Austria.

All test compounds were synthesized in the Laboratory of Oxylipins of Shemyakin-Ovchinnikov Institure of Bioorganic Chemistry, RAS, Russia as previously described (1) and stored only as ethanol or dimethyl sulfoxyde (DMSO) stocks under argon atmosphere at –52˚C.

All cell lines were maintained at 37˚C under 5% CO_2 in the medium (see below), supplemented with 2 mM L-glutamine, 25 mM HEPES (9), 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. MCF-7 and HEK-293 were cultured in DMEM medium, IMR-32 in Eagle's MEM medium

Table I. *Cytotoxic and anti-proliferative action of N-acyl dopamines on various cell lines.*

Dopamine amide of fatty acid	Test	Cell line, LD_{50} (μM)			
		K-562	MCF-7	HOS	IMR-32
Arachidonic	LDH	43±17*	59±10	20±2*	6±2*
	MTT	5±3	57±11	11±2	0.7±0.2
Oleic	LDH	44±20*	87±6*	18±1*	8±1*
	MTT	4±2	71±4	8±2	0.9±2
Docosahexaenoic	LDH	20±4*	90±10*	26±2*	3±1*
	MTT	16±3	48±6	9±2	0.6±0.2
Stearic	LDH	>100*	>100	16±1	7±1*
	MTT	36±8	>100	15±4	1.5±0.1

*Statistically significant difference between LDH and MTT test data.

Table II. *Anti-proliferative and cytotoxic action of the active N-acyl-dopamines-related compounds on various cell lines.*

Arachidonic acid amide of	Test	Cell line, LD_{50} (μM)	
		HOS	IMR-32
3-O-methyl dopamine	LDH	55±10*	78±8*
	MTT	29±1	39±9
Tyramine	LDH	39±1*	49±5
	MTT	28±1	43±7
Epinephrine	LDH	31±10	4±1*
	MTT	20±5	1.2±0.5
Norepinephrine	LDH	28±1	23±4*
	MTT	22±8	41±4

*Statistically significant difference between LDH and MTT test data.

supplemented with 1 mM sodium pyruvate, while Namalwa and K-562 were grown in RPMI 1640 medium. The medium for MCF-7 and HEK-293 cells was additionally supplemented with 1% non-essential amino acids solution. Cells were passaged each 3 days and continiously grown for no more than 40 passages. Attached cells were detached with 0.25% trypsin in 0.53 mM EDTA in Hanks' salts. Cells were counted using a glass hemocytometer.

For analysis of toxicity of NADA and related compounds, cells were plated in 96-well plates at a density of 1.25×10^4 cells per well and grown for 3 days. Serial dilutions of test compounds (the range of final concentrations after addition to cells of 0.1-100 μM) prepared in DMSO and dissolved in the culture medium were added to the cells in triplicate for each concentration and incubated for 18 h. The incubation time was chosen on the basis of the most pronounced differences between the compounds tested. The final DMSO concentration was 0.5%. Negative control cells were treated with 0.5% DMSO. Positive control cells were treated with 3.6 μl of 50% Triton X-100 in ethanol per 200 μl of cell culture medium. Separate controls were without DMSO (no difference with the control 0.5% DMSO was found, data not shown). The effect of test substances on cell viability was evaluated using both MTT test (based on the MTT dye reduction by mitochondria of living cells) (13) and lactate dehydrogenase (LDH) test (based on the lactate dehydrogenase liberation from the dead cells) (14).

For the MTT test, after removal of the medium with the test compounds, cells and controls were incubated for 1.5 h with 0.5 mg/ml of MTT in Hank's solution, supplemented with 10 mM of D-glucose. After this incubation, the solution was removed and the cells were dissolved in DMSO. For the suspension cells, 10× stock of MTT dye in Hank's solution was added directly to the medium with the test compounds; after the incubation, an equal volume of 0.1 N HCl in isopropanol was added to the medium to dissolve the formazan crystals formed by the cells from the MTT. In this case, we also used a separate control with substances in the medium without cells to compensate for the interaction of NADA with the MTT dye. The amount of the reduced dye was determined colorimetrically at 594 nm with a reference wavelength 620 nm using an EFOS 9505 photometer (Sapphire, Moscow, Russia). Additionally, the attachment and cell shape of adherent cells were evaluated microscopically.

For the analysis of the LDH activity, to a 60 μl aliquot of the cell medium 1 μl of 50% Triton X-100 in ethanol, 8 μl of 36 mg/ml DL-lactate in Tris-HCl pH 8.5, 8 μl of 2 mg/ml INT and 8 μl of 3 mg/ml NAD^+ mixed with 13.5 U/ml diaphorase were sequentially added. The last two compounds were dissolved in phosphate-buffered saline with 1.2% sucrose and 0.03% bovine serum albumin prior to each experiment. INT stock solution (10×) was prepared in DMSO and stored at –20˚C. The reaction mixture was incubated for 20 min at room temperature after which the reaction was terminated with 5 μl of 3N HCl. Its optical density was determined at 490 nm using an EFOS 9505 photometer (Sapphire). Cell medium with purified lactate dehydrogenase (0.05 U/ml) was incubated with the test substances as a separate control; neither one inhibited the enzyme in the concentration range used.

Each experiment was repeated three times. Curves for calculating the 50% lethal dose (LD_{50}) were generated using the GraphPad Prism software (www.graphpad.com); the substances that caused ≤10% cell death at 100 μM were considered inactive. Data are presented as mean±standard deviation. Data were compared using the unpaired Student's *t* test; *p* values of 0.05 were considered significant.

Results

We tested the cytotoxic and anti-proliferative action on cancer cell cultures of four N-acyl dopamines: dopamine amides of docosahexaenoic (DHA-DA), arachidonic (AA-DA), oleic (Ol-DA) and stearic (St-DA) acids, as well as several related compounds: arachidonic acid amides of 3-O-methyldopamine (AA-3MDA), tyramine (AA-TA), epinephrine (AA-AD), 3-O-sulphodopamine (AA-DA-SO4), norepinephrine (AA-NOR) and dopamine amide of prostaglandin E_2 (PGE2-DA). The substances in the N-acyl dopamines group were chosen for their endogenous occurence (3-5), while the palette of related compounds was designed to include their most probable endogenous metabolites and precursors (7). We also included dopamine (DA) in our research as a control. The highest concentration used was 100 μM.

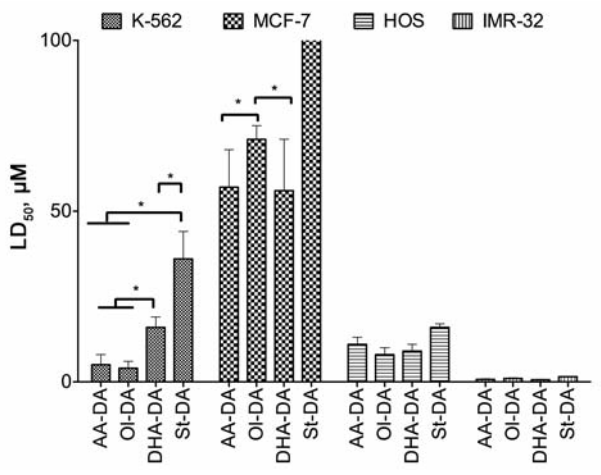

Figure 1. *Cytotoxicity of dopamine amides of arachidonic (AA-DA), docosahexaenoic (DHA-DA), oleic (Ol-DA) and stearic (St-DA) acids for various cell lines. MTT test data for 18-h incubation time, LD_{50}, mean±standard deviation; *statistically significant difference, $p<0.05$ (multiple ANOVA with the Tukey post test)*

To investigate the spectrum of NADA cytotoxicity we have chosen 5 cancer cell lines representing almost all major histological cancer types (15), except germinoma: lymphoma (Namalwa), leukemia (K-562), carcinoma (MCF-7), sarcoma (HOS) and blastoma (IMR-32); as a comparison, we used one non-malignant cell line (HEK-293) (16).

Free dopamine was absolutely inactive up to 100 μM for all cell lines tested. All N-acyl dopamines were cytotoxic for K-562, MCF-7, HOS and IMR-32 cell lines with LD_{50} values in a range from 0.6 to 71 μM (Figure 1, Table I). N-acyl dopamines also exhibited anti-proliferative action for the Namalwa cell line with IC_{50} 8±1, 18±5, 8±3 and 7±3 μM for AA-DA, Ol-DA, DHA-DA and St-DA, respectively. However, these substances were only slightly toxic for the non-malignant HEK-293 cell line with LD_{50} >100 μM and cell survival at 100 μM of substance ranging from 60 to 90%.

N-acyl dopamine derivatives and biosynthetic precursors were less active than the parent N-acyl dopamine (Table II). Sulfated AA-DA derivative was absolutely not toxic for all cell lines tested. N-arachidonoyl tyramine, 3-O-methyl dopamine and norepinephrine were only minimally toxic for the HEK-293, K-562 and MCF7 cell lines with LD_{50} values >100 μM. The only metabolite for these cell lines with a LD_{50} value <100 μM was N-arachidonoyl epinephrine (LD_{50} values 75±16, 19±3 and 44±10 μM, respectively).

N-arachidonoyl tyramine exhibited a LD_{50} value below 100 μM only for HOS and IMR-32 cells. Dopamine amide of prostaglandin E_2 was toxic only for the IMR-32 cell line with LD_{50} in MTT and LDH assays of 11±2 μM and 32±3 μM, respectively; for the other cell lines, it reduced proliferation by ≤20% at the maximal concentration.

Discussion

The anti-proliferative action of N-acyl dopamines on cancer, as well as normal cell lines, is known (9-11, 17, 18) from studies that have used N-arachidonoyl dopamine or its synthetic analogues only. In addition, selective cytotoxic action of dopamine and related amides of an exogenous piperic acid is known (19). This work, which is the first systematic study of cytotoxicity of endogenous acyl dopamines, has included a set of known or proposed metabolic precursors and derivatives of the aforementioned lipids in order to check whether such transformation could decrease activity and, thus, represent a possible way for cancer cells to escape toxicity.

The presented data demonstrated that all NADA were able to induce cell death in all cancer cell lines tested, except for St-DA, which was inactive at MCF-7 and, partially, at K-562 cells. There was no universal dependence on the structure of the fatty acid residue; however, Ol-DA and DHA-DA were the leaders, causing death with the lowest LD_{50} values in 3 of 5 cell lines each (Table I, Figure 1).

HEK-293 cells were resistant to NADA's action up to 100 μM. It could be hypothesized, that the higher resistance of this cell line towards N-acyl dopamines was due to their different origin: this line is not truly a cancerous one (20) and, thus, could lack certain metabolic properties, typical for highly mutated cancerous cells.

The comparison of toxicity data between NADA and modified NADA molecules both in the fatty acid moiety and in dopamine residue (Table III) showed that there are two ways to dramatically decrease the cytotoxicicty of a NADA molecule: the transformation of the polyunsaturated fatty acid residue to a prostaglandin E_2 form and a modification of the catechol group, especially with a large hydrophilic sulfonic acid residue.

A typical property of almost all substances tested was a higher value of LD_{50} in the LDH test in comparison to the MTT test. As far as MTT reduction is a measure of the cell mitochondria and anti-oxidative system state (21) and LDH release indicates cell membrane disruption (21), it may be speculated that NADA and related compounds primarily disrupt the energy metabolism of the cell and do not cause immediate cell lysis. This is consistent with the observed caspase 3 activation during NADA-induced cell death of glioma cells (9), which is a hallmark of the mitochondria-dependent apoptosis induction (22), as well as with the overall concept of vanilloid-induced apoptosis (23). Preliminary data supporting this assumption have been already obtained by our group (24).

N-arachidonoyl epinephrine has demonstrated two unusual properties in comparison to other substances tested. First, its LD_{50} value was much lower in the LDH, and not in the MTT, test, which possibly could be an indication of some peculiar interaction with the cell's oxidative system causing

Table III. *Comparative anti-proliferative activity of natural N-acyl dopamines for various cell lines. LD_{50} representation, -; >100 μM; +, 50-100 μM; ++, 10-50 μM; +++, <10 μM. NT, not tested.*

Cell line	Compound									
	AA-DA	DHA-DA	Ol-DA	St-DA	AA-3MDA	AA-TA	PGE2-DA	AA-NOR	AA-AD	AA-DA-SO4
IMR-32	+++	+++	+++	+++	++	+	++	++	+++	-
MCF7	+	+	+	-	-	-	-	-	+	-
Namalwa	+++	+++	++	+++	-	-	-	NT	-	-
K-562	+++	++	+++	++	-	-	-	-	++	-
HOS	++	+++	+++	++	++	++	-	++	++	-
HEK-293	-	-	-	-	-	-	-	-	+	-

the latter to increase MTT reduction. This interaction could be the key to the second property of this compound, *i.e.* the ability to induce cell death in HEK-293 cells; however, the details of this effect require further studies.

In conclusion, we found NADA to be able to induce cell death in most histological cancer types studied; to be able to do this a NADA molecule had to have an intact catechol group and a hydrophobic fatty acid residue. Taken together with the ability of these molecules to protect normal neuronal cells against stressful conditions, this may be an indication that NADA may be novel effector molecules of the organism's cancer defence system. It is tempting to speculate, that NADA may be even a component of a stand-alone lipid anticancer sub-system; however, this hypothesis requires further experimentation.

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