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Acylamido analogs of endocannabinoids selectively inhibit cancer cell proliferation

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ABSTRACT

A series of amide derivatives of long-chain fatty acids has been studied for their effects on the proliferation of cancer cells in vitro. Fatty acids ranged from palmitic to higher polyunsaturated types containing 22 carbon atoms. The amino portions of the molecules included ammonia, ethanolamine, various amino acids and dopamine. Several cell lines were used as models and these included HTB-125 (normal human breast cells), HTB-126 (human breast cancer cells), HeLa (cervical cancer cells), WI-38 (human embryonic lung cells), RAW264.7 (mouse macrophage tumor cells) and RBL-2H3 (rat basophilic leukemia cells). The HTB lines were obtained from the same donor, so, could be considered a matched pair, that is, normal control versus cancer cells and thus, provide a model for testing specificity of action for the acylamido analogs. While many compounds were efficacious in inhibiting the proliferation of various cell lines, only two analogs showed a high degree of specificity in the matched HTB cell lines. *N*-palmitoyl dopamine and *N*-palmitoyl tyrosine each demonstrated complete specificity of action at a concentration of 10 μM and were highly efficacious in both cases. No clear structure–activity pattern could be derived from these studies since the intensity of the inhibitory action seemed to depend on three factors, namely, the fatty acid, the amine group and the cell type.

1. Introduction

The discovery of an endogenous cannabinoid system (ECS) has fueled an intense interest in identifying the roles that this complex may have in the regulation of cell function and the possible significance for health care strategies.[1] Various studies have addressed this question in both physiological and pathological processes at central as well as peripheral sites. Thus far, the endocannabinoid system has been shown to be involved in the immune system, energy balance, stress recovery, food intake, metabolic homeostasis, cardiovascular depressive effects, endocrine axes, fertility, pain modulation and neuroprotection. The ECS may also have a role in cancer biology because of its effects on cell proliferation.[2]

Many of the substances, and related analogs, that modulate this system are lipid in nature and generally are comprised of a long-chain fatty acid coupled to a more polar group such as ethanolamine, glycerol, dopamine or one of a number of amino acids (Fig. 1). The fatty acid component of the structure can range from a fully saturated acid such as palmitic to a higher polyunsaturated type such as arachidonic. To date, fairly extensive groups of these ligands or mediators have been identified and to some degree characterized.[3] Thus, it is probably safe to say that the extent of this family of compounds is far greater than what has thus far been discovered.

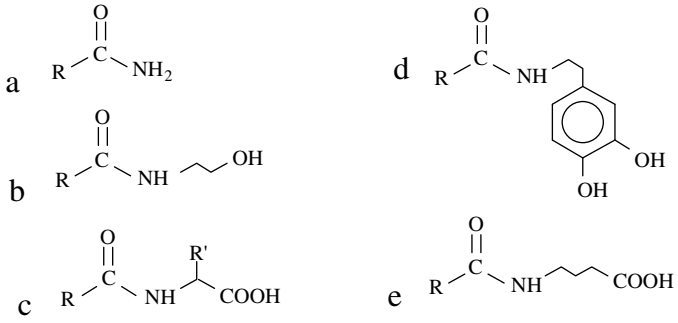

Figure 1. Chemical structures. (a) *N*-acyl amides. (b) *N*-acyl ethanolamides. (c) *N*-acyl amino acids (elmiric acids) R′ = H, CH_3, (d) *N*-acyl dopamine. (e) *N*-acyl GABA (γ-aminobutyric acid).

Our own interest is focused primarily on the amino acid conjugates that we have named elmiric acids (EMA).[4] These analogs do not produce typical cannabimimetic actions in animal models, however, they do show both analgesic[5–7] and anti inflammatory[8] properties in vivo. In addition, they exhibit a number of in vitro

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properties such as inhibition of fatty acid amide hydrolase (FAAH),[9] effects on prostaglandin profiles,[4] calcium mobilization and firing rates in dorsal root ganglia,[10,11] and cell proliferation.[4]

The plant derived cannabinoids, or phytocannabinoids, have been studied as possible anti cancer agents for some time.[12] Various mechanisms have been suggested to help explain their anti proliferative actions and these may have relevance for the effects of the endocannabinoids as well.[13–16] Tetrahydrocannabinol (THC), the principle psychotropic ingredient in *Cannabis*, has long been used in the form of the drug dronabinol, to reduce the side effects of chemotherapy, but, its impact on the course of the disease in humans is not clear. Synthetic analogs of THC have been prepared and studied for a possible use in cancer therapy. Such a compound, ajulemic acid (AJA) is a peripheralized,[17] non psychoactive analog with moderate anti cancer actions in preclinical models.[18] Nabilone, another THC analog, is used clinically to control nausea and emesis in patients receiving chemotherapy, however, its possible effects on tumor progression has not been reported.[19]

We previously carried out a limited study on the inhibition of proliferation by several EMAs in a mouse macrophage tumor-derived cell line, RAW264.7.[8] Full dose–response data were not obtained in that study; however, a complete inhibition of proliferation was seen between 1 and 10 μM. Only the effects of glycine and alanine conjugates were reported and their polyunsaturated derivatives appeared to be more active than the saturated analogs. The limited nature of the study did not allow any detailed conclusions regarding structure–activity relationships (SAR) to be made.

In subsequent experiments reported here, we sought to extend the previous work to a series of new analogs and to expand the number and variety of cell types studied with these compounds. In addition, more complete relationships between drug concentration and cell proliferation have been obtained. Of particular interest, is an observed selectivity of inhibition when the effect on tumor cells is compared with a matched normal control, which was observed for certain compounds in a model of human breast cancer.

2. Chemistry

Only one of the compounds used in this study, *N*-arachidonoyl-L-tyrosine; L-EMA-10 (16:0) (see Ref. 8 for nomenclature), was synthesized in the university laboratories using the procedures described below in Section 6.1.2. Briefly this involved condensation of the mixed anhydride palmitoyl-*i*-butylchloroformate with L-tyrosine methyl ester in the presence of triethylamine followed by saponification with lithium hydroxide to yield the EMA. The rest of the compounds came from a commercially available library of endocannabinoids obtained from Biomol International LP (Plymouth Meeting PA 19462) that contains sixty examples, half of which would fall into the category of EMAs. They are composed of glycine, L-alanine and γ-aminobutyric acid (GABA) conjugates with ten different naturally occurring long-chain fatty acids ranging from palmitic to docosahexaenoic acid. Also included are sets of simple amine, ethanolamine and *N*-dopamine derivatives of the same fatty acids.

3. Results

3.1. Time course

In Figure 2 the time course for the anti proliferative effect of one of the EMAs, *N*-palmitoyl tyrosine (10 μM) was compared to vehicle (DMSO) treated cells and AJA (10 μM), a cannabinoid analog with anti tumor activity.[18] Equal numbers (2000 cells/well) of human tumor-derived breast cancer cells (HTB-126) were plated at time zero. All three treatments were applied at 24 h and cell numbers measured two hours later. Subsequent measurements, on parallel plates, were made at 48, 72 and 96 h post plating time. The EMA showed an immediate anti proliferative effect whereas the AJA showed no effect initially. At 48 and 72 h the two agents produced the same response while at 96 h the beginning of a divergence was observed in which the inhibitory effect of AJA appeared to be increasing.

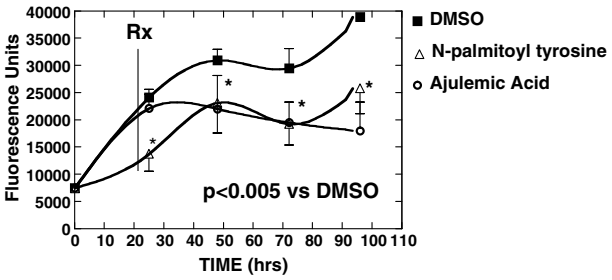

Figure 2. Time course in HTB-126 breast cancer cells. Cells derived from a human tumor were obtained from ATCC and seeded in 18 wells of four 24 well plates at 2000 cells/well under the conditions described in Section 6.1.1. Following a 24 h incubation period, each plate was treated with vehicle, *N*-palmitoyl tyrosine and AJA ($N = 6$). After 60 min, one plate was subjected to the cell proliferation assay (see Section 6.1.1), a second plate was assayed after 24 h, a third after 48 h and the last after 72 h. Both agents were given at a final concentration of 10 μM from a 100× stock solution in DMSO. Values shown are in relative fluorescence units.

3.2. Selective inhibition of human breast cancer cells (HTB-126) by acylamido analogs

Figure 3(A–E) shows dose–response data for several acylamido analogs in which the effects on human tumor-derived breast cancer cells (HTB-126) were compared with normal human breast cells (HTB-125). The best separation of activity was obtained with *N*-palmitoyl dopamine (Fig. 3A), which had no effect on the normal (HTB-125) cells at concentrations up to 10 μM but produced an 80% inhibition of tumor cell (HTB-126) proliferation. The other dopamine analogs were also inhibitory under the same conditions, however, they did exhibit moderate anti proliferative effects on normal cells (Fig. 3B–D).

N-palmitoyl tyrosine, an example of the EMA family, gave a response pattern similar to that of *N*-palmitoyl dopamine in the same model (Fig. 3A and E). Here, a vigorous inhibition of tumor cell proliferation was seen at 10 μM with no changes at 0.1–1.0 μM concentrations, similar to the dopamine analog responses. All of the values presented in Figure 3A–E were normalized to 100% for the vehicle treated controls.

Some insight into the mechanism of action of *N*-palmitoyl tyrosine was obtained using a receptor antagonist approach. Panel F shows the effect of a CB1 antagonist, rimonabant (SR141716), on the anti proliferative response of *N*-palmitoyl tyrosine in HTB-126 tumor cells. The profound decrease in proliferation caused by treatment with *N*-palmitoyl tyrosine (10 μM) was totally reversed by rimonabant (10 μM). The antagonist alone produced a small increase in tumor cell number. The values in this panel are given as fluorescence units, which are directly proportional to cell number.

3.3. Effects of acyl dopamide analogs on proliferation of cervical cancer (HeLa) cells

The anti proliferation effects of a series of dopamine-unsaturated fatty acid conjugates in a different tumor cell type was studied and the results are shown in Figure 4. In cervical cancer (HeLa)

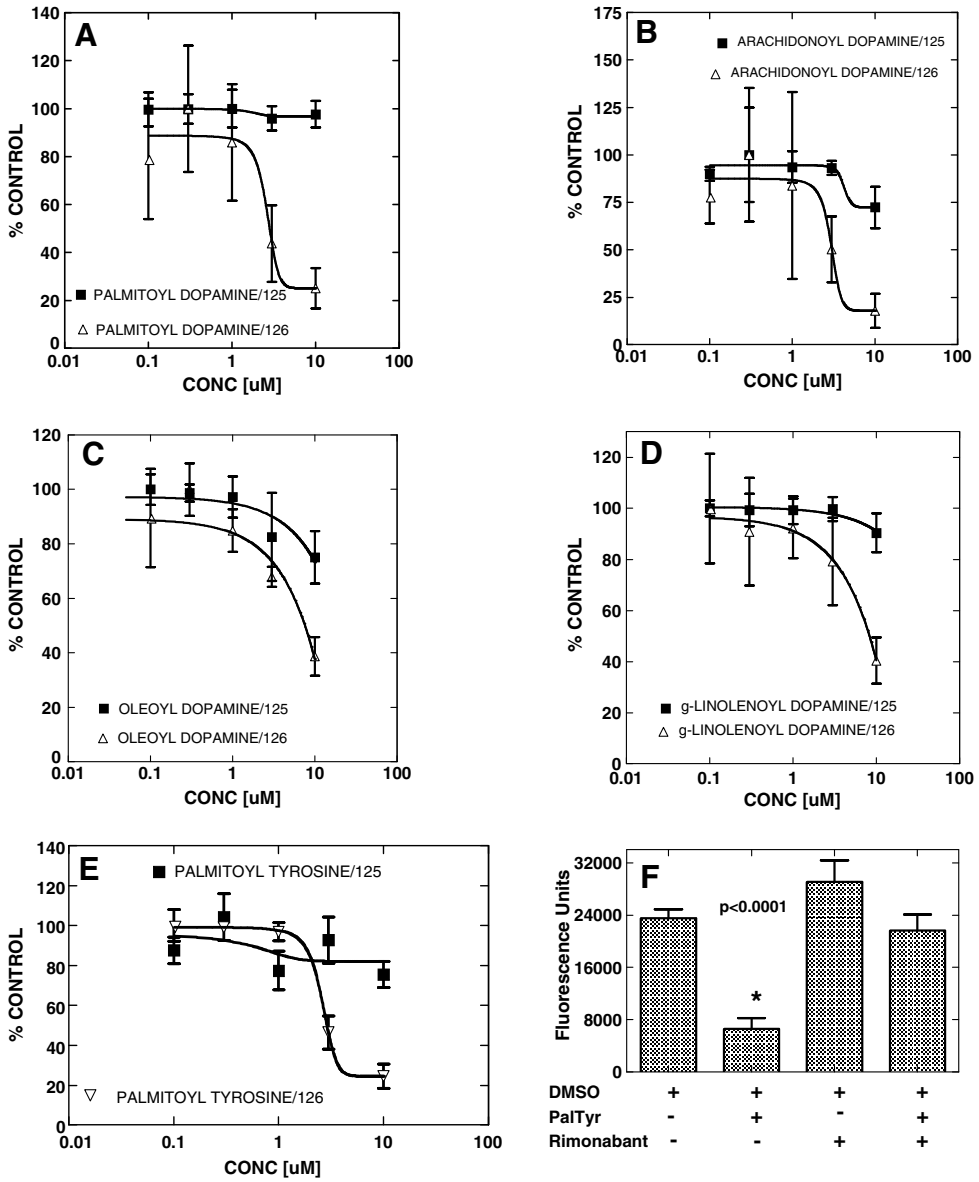

Figure 3. Selective inhibition of human breast cancer cells (HTB-126) versus normal (HTB-125) cells by acylamido analogs. Cells were grown and treated under conditions similar to those described in Figure 2 except that 96 well plates were used and four replicates were done for each point. The cell proliferation assay was performed at 48 h following drug treatment and the values in (A–E) are expressed as a percentage of the vehicle treated wells. In (F), the antagonist rimonabant (10 μM) was added 30 min prior to the treatment with *N*-palmitoyl tyrosine (10 μM) and the assay performed 48 h later. Here the values are shown as fluorescence units since there are two control treatments.

cells, all of the analogs tested produced strong inhibition at 10 μM concentrations. A tendency toward stimulation was seen at lower concentrations analogous to the *N*-palmitoyl tyrosine effect shown in Figure 3E. A negative control agent, *N*-palmitoyl glycine, had no effect as expected and a positive control, AJA, appeared to be less active than the dopamine derivatives, however, this may be due to a time course effect (see Fig. 2).

3.4. Effects of acylamides on human embryonic lung (WI-38) and mouse macrophage tumor (RAW264.7) cell proliferation

The simplest examples of acylamido analogs are those where the polar group is NH_2. In Table 1 are data from a single concentration (10 μM) treatment of human embryonic lung fibroblasts and mouse macrophage cancer cells with a series of such fatty acid amides ranging from palmitamide to docosahexaeneamide. None of the analogs was particularly effective in either cell type, thus, there were no clear structure–activity trends.

A similar study was done with a possibly more relevant series of analogs in which the polar group is ethanolamine (Table 2). Again, in these two cell types most of the compounds were not especially potent with one possible exception, namely, docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide in the WI-38 model. Not shown are the results of a very similar experiment done in the same models where a series of *N*-fatty acid GABA conjugates were tested. In almost every case, modest stimulatory actions were seen with one exception. *N*-oleoyl GABA produced a small decrease in cell proliferation in RAW cells.

3.5. The effects of *N*-acyl dopamide conjugates on human embryonic lung (WI-38) and mouse macrophage tumor (RAW264.7) cell proliferation

One other class of compounds, the *N*-fatty acid dopamine type, was studied in the WI-38/RAW cell model and the findings are shown in Table 3. Here, in almost every example, a robust

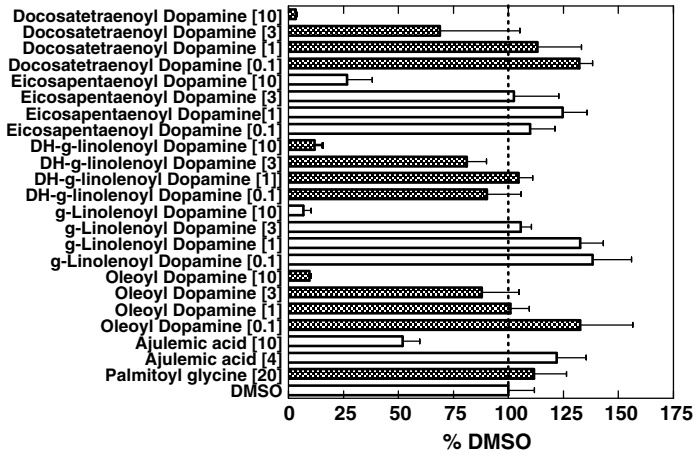

Figure 4. Effects of acyl dopamide analogs on proliferation of cervical cancer (HeLa) Cells. The cells were seeded in 96 well plates and cultured as described in Section 6.1.1. Treatments were made at 24 h after seeding and the assays performed at 48 h post treatment. Four replicates were done for each and the concentrations are shown as the numbers in brackets in μM units. DH = dihomo. Values are mean ± SEM.

Table 1
Effects of acylamides on human embryonic lung (WI-38) and mouse macrophage tumor (RAW264.7) cell proliferation.

Treatment (c = 10 μM)	WI-38 cells		RAW cells	
	Control (%)	SD	Control (%)	SD
DMSO	100	17	100	16
Palmitamide	118	17	100	7.3
Oleamide	94	22	134	12
Linolenamide	85	35	126	28
γ-Linolenamide	33	14	94	13
Eicosa-1Z,14Z-dienamide	55	18	74	7.5
Dihomo-γ-Linolenamide	58	7.6	82	9.5
Arachidonamide	58	17	91	10
Eicosapentaenamide	61	30	64	13
Docosatetra-7Z,10Z,13Z,16Z-enamide	61	23	91	10
Docosahexaenamide	89	15	112	22

Cell culture conditions and treatment procedures are described in Section 6.1.1 and in Figure 3.

Table 2
Effects of acylethanolamides on human embryonic lung (WI-38) and mouse macrophage tumor (RAW264.7) cell proliferation.

Treatment (c = 10 μM)	WI-38 cells		RAW cells	
	Control (%)	SD	Control (%)	SD
DMSO	100	17	100	16
Palmitoyl ethanolamide	130	32	66	5.2
Oleoyl ethanolamide	140	31	117	23
Linoleoyl ethanolamide	63	16	100	10
γ-Linoleoyl ethanolamide	54	23	87	23
Eicosa-1Z,14Z-dienoyl ethanolamide	58	13	54	21
Dihomo-γ-linolenoyl ethanolamide	54	6.5	70	30
Arachidonoyl ethanolamide	74	38	110	35
Eicosapentaenoyl ethanolamide	92	27	83	18
Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide	24	7.4	65	11
Docosahexaenoyl ethanolamide	50	17	36	15

Cell culture conditions and treatment procedures are described in Section 6.1.1 and in Figure 3.

inhibition of cell proliferation was found in both cell lines. Thus, as a group, the dopamine conjugates were more active than any of the others described in this report. Indomethacin has been reported to inhibit glioma cell proliferation,[20] and was included here as a positive control. However, it actually caused an increase in both cell types suggesting that it may be very tissue specific in its possible anti cancer activity.

3.6. Effects of palmitoyl and arachidonoyl derivatives on LPS-stimulated, rat basophilic leukemia (RBL-2H3) cell proliferation

Finally, a study was done using rat basophyllic leukemia cells (RBL-2H3) with examples of both EMAs and *N*-acyl dopamine analogs (Table 4). The amino acid groups consisted of glycine, L-alanine and GABA while in each case palmitoyl and arachidonoyl fatty acid groups were included. All cells were stimulated with lipopolysaccharide (LPS) (10 ng/ml). Concentrations of 0.1, 1.0 and 10 μM were used for each compound; the results are shown both as unprocessed data and also as the ratio of treated over control values. All of the compounds except for *N*-palmitoyl glycine, which had no inhibitory action, were effective at 10 μM and both of the dopamine derivatives seemed to be more potent than any of the EMAs.

4. Discussion

Almost a century has passed since Paul Ehrlich used the term "silver bullet" to describe salvarsan, his drug for the treatment of syphilis. Unfortunately, salvarsan did not exhibit the specificity of action he sought, but this has not discouraged generations of scientists since then from continuing the search for agents that will only target disease sites leaving healthy tissue unaffected. This has been particularly true in the area of cancer chemotherapy where specificity of action remains a principle consideration in designing anti cancer agents. In this report, we describe a family of molecules, the acylamido analogs of the endocannabinoids, which may provide an approach for advancing toward this goal.

In an earlier study, we presented preliminary findings showing that a subset of the acylamido analogs called EMAs inhibited the proliferation of the RAW264.7 murine leukemia virus-induced tumor cell line.[4] In that report, a number of the EMAs in the glycine and L-alanine groups showed a profound inhibition of RAW cell proliferation at a 10 μM concentration. These observations prompted us to carry out further experiments to better characterize this effect and to examine the possibility of selectivity between normal and cancer cells. It was also decided to look beyond the EMAs and include other analogs (Fig. 1), for example, the *N*-dopamine-fatty acid derivatives, because of reports on their effects in cancer cells.[21–23]

To test for selectivity, we utilized two commercially available cell lines (HTB-125 and HTB-126) that were derived from human breast tissue (see Section 6.1.1 for details). The former was

Table 3
The effects of *N*-acyl dopamide conjugates on human embryonic lung (WI-38) and mouse macrophage tumor (RAW264.7) cell proliferation.

Treatment (c = 10 μM)	WI-38 cells		RAW cells	
	Control (%)	SD	Control (%)	SD
DMSO	100	18	100	26
Palmitoyl dopamine	41.6	9.5	11.1	1.4
Oleoyl dopamine	24.0	5.5	7.02	0.61
Linoleoyl dopamine	21.6	15	7.39	7.1
γ-Linoleoyl dopamine	38.3	25	6.10	1.0
Eicosa-1Z, 14Z-dienoyl dopamine	66.6	26	3.95	0.93
Dihomo-γ-linolenoyl dopamine	25.5	10	5.72	0.94
Arachidonoyl dopamine	22.1	6.6	2.33	0.87
Eicosapentaenoyl dopamine	48.0	23	10.5	12
Docosatetra-7Z,10Z,13Z,16Z-enoyl dopamine	25.7	9.5	4.32	0.92
Docosahexaenoyl dopamine	40.7	23	6.60	0.77
Indomethacin	144	32	177	35

Cell culture conditions and treatment procedures are described in Section 6.1.1 and in Figure 3.

Table 4
Effects of *N*-palmitoyl and *N*-arachidonoyl derivatives on rat basophilic leukemia (RBL-2H3) cell proliferation.

Treatment (n = 3)	Concn (μM)	Response	SD	Control (%)
DMSO	—	4665	601	100
Palmitoyl glycine	0.1	5906	611	130
Palmitoyl glycine	1.0	5346	1246	110
Palmitoyl glycine	10	5291	948	110
Arachidonoyl glycine	0.1	6224	524	**130**
Arachidonoyl glycine	1.0	6203	1016	**130**
Arachidonoyl glycine	10	959	392	**21**
Palmitoyl L-alanine	0.1	5067	1866	110
Palmitoyl L-alanine	1.0	4355	1005	90
Palmitoyl L-alanine	10	614	35	10
Arachidonoyl L-alanine	0.1	5822	819	**120**
Arachidonoyl L-alanine	1.0	7315	1454	**160**
Arachidonoyl L-alanine	10	2312	1042	**50**
Palmitoyl GABA	0.1	5459	1544	120
Palmitoyl GABA	1.0	4636	732	100
Palmitoyl GABA	10	4461	151	100
Arachidonoyl GABA	0.1	4531	808	**100**
Arachidonoyl GABA	1.0	5605	1048	**120**
Arachidonoyl GABA	10	674	30	**15**
Palmitoyl dopamine	0.1	5882	658	130
Palmitoyl dopamine	1.0	2365	696	50
Palmitoyl dopamine	10	704	13	15
Arachidonoyl dopamine	0.1	5254	601	**110**
Arachidonoyl dopamine	1.0	3447	670	**70**
Arachidonoyl dopamine	10	648	4	**14**

Ninety-six-well plates with 500 mast cells/well/100 μl media with serum were incubated for 24 h. Media were then changed to 100 μl of *serum free* RPMI and incubated for 4 h. Cells were treated as indicated in the table and incubated for 1 h. LPS (10 ng/ml) was then added to each well and the incubation continued for 24 h. Cell numbers were then obtained using the CellTiter-Glo assay.

obtained from healthy breast tissue while the latter was derived from a breast tumor present in the same donor. We reasoned that this would provide a good model to test our analogs for selective action where most extraneous factors would be eliminated. The optimal time point for measuring changes in proliferation was found to be not less than 48 hours as shown in Figure 2. In this experiment *N*-palmitoyl tyrosine, an example of an EMA, was compared with a positive control substance, AJA that has anti cancer activity.[18] In passing, it is interesting to note that although efficacies between the two were similar, the onset of action was different suggesting the possibility of different mechanisms.

Five analogs were tested using this breast cancer model and the findings are shown in Figure 3A–E. The results ranged from virtually no selectivity with *N*-oleoyl dopamine (Fig. 3C) to completely selective effects with *N*-palmitoyl dopamine (Fig. 3A) and *N*-palmitoyl tyrosine (Fig. 3E). All of the data shown were normalized to the control values obtained from vehicle treated cells under identical conditions. Based on these limited data, we estimate a rank order of selective inhibition to be: *N*-palmitoyl dopamine = *N*-palmitoyl tyrosine > *N*-arachidonoyl dopamine = *N*-γ-linoleoyl dopamine > *N*-oleoyl dopamine. While no clear SAR can be discerned from this small group of compounds, it seems reasonable to conclude that selective action can be achieved with palmitoyl derivatives. It would be of great interest to see whether similar results would be obtained in other matched models of anti cancer activity. Moreover, these observations suggest that some of the acylamido analogs would show a high therapeutic index in vivo.

The question of receptor mediation in the above experiments has been briefly addressed and the results are shown in Figure 3F. Using the same model for which the other data in Figure 3 were obtained, the effect of pretreatment with a CB1 receptor antagonist, rimonabant[24,25] was studied. A complete reversal of the inhibition of proliferation by *N*-palmitoyl tyrosine was found suggesting that the CB1 receptor has an important role in this process. There are no data on the levels of CB1 in either of these breast tissue-derived cell lines, however, it is interesting to note that a recent report gives data that cancer cells over express both CB1 and CB2 receptors and that receptor activation inhibits proliferation.[26,27] A greater expression level of CB1 in HTB-126 cells versus HTB-125 cells might help to explain the selectivity found in our study. Since *N*-arachidonyl glycine has been reported to have no affinity for CB1,[28] it may be inferred that *N*-palmitoyl tyrosine likewise does not bind. Thus, our observation with rimonabant could be the result of an indirect CB1 activation mechanism through some type of "cross talk" effect involving a different receptor such as GPR18[29] for which *N*-arachidonoyl glycine is a ligand (vide infra).

Several other agents were tested for their effects on the breast cancer model (unpublished observations). A second CB1 antagonist, AM251, completely reversed the anti proliferative effect of 10 μM *N*-palmitoyl tyrosine in agreement with the data shown in Fig. 3F. However, the CB2 antagonist, AM630, gave a weaker reversal of the *N*-palmitoyl tyrosine response suggesting a lesser role for CB2 in our model. A possible role for PPAR-γ was investigated since AJA has been reported to bind and activate this important nuclear receptor.[30,31] GW9662, a potent, selective and irreversible PPAR-γ antagonist had no effect on *N*-palmitoyl tyrosine inhibition in our model suggesting the absence of a role for this nuclear receptor. Finally, the cells were treated with a competitive inhibitor of FAAH, *N*-arachidonoyl serotonin, since other EMAs were shown to inhibit this catalyst of endocannabinoid metabolism[9] and an elevation of anandamide levels could, in some cases, result in a decrease in cell proliferation. However, no effect was found compared with vehicle treated cells suggesting that the observed inhibitions were not due to an increase in endocannabinoid concentration in HTB-126 cells. On the other hand, direct treatment with anandamide at 10 μM caused a significant 38% ($p < 0.006$) decrease in HeLa cell proliferation pointing to possible differences in mechanism in other cell types. Thus, the question of involvement of FAAH in the anti proliferative actions of the EMAs remains open.

Another possible mechanism needs to be considered in future studies. The orphan receptor GPR 18 has recently been shown to be a target for *N*-arachidonoyl glycine, the prototypic EMA.[29] It was reported that this receptor is abundant in monocytic and lymphoid cell lines, and was increased after stimulation with the mitogenic substance phytohemagglutinin. If GPR 18 is also expressed in breast cancer cells, it might have a role in the action of the acylamido analogs on cell proliferation and provide the basis for a putative mechanism.

In addition to the subject of selective actions for the acylamido analogs, the scope of the inhibitory effects in terms of cell types

and molecular structure has been studied. Figure 4 and Tables 1–4 address these questions. A series of dopamine-fatty acid analogs was studied in cervical cancer cells (HeLa) and the results are shown in Figure 4 in which *N*-palmitoyl glycine, a saturated fatty acid analog, and AJA are the controls as well as vehicle treated cells. Concentrations of 1, 3, and 10 μM were used for each of the analogs all of which were unsaturated fatty acid derivatives. Significant inhibition of proliferation was seen at the highest concentration for each compound similar to the data shown for the HTB-126 cells (Fig. 3). In each case, at 3 and 1 μM no significant inhibition and even trends toward stimulation of cell proliferation were observed indicating a steep dose–response curve. Since no matched normal control cells are available for HeLa cells, it was not possible to study the selectivity of these compounds in this model. Nevertheless, the data suggest that these analogs may be effective in more than one type of cancer cell. Our findings are in agreement with an earlier report on a series of acyl dopamides in the MCF-7 human breast cancer cell line.[21] The authors identified *N*-arachidonoyl dopamine (NADA) as the most active derivative and showed that its anti proliferative action is mediated by CB1.

Structurally, the simplest acylamido analogs are the fatty acid amides (Fig. 1a) that we have studied in two different cell lines (Table 1). The WI-38 human embryonic lung cell is a rapidly proliferating line with fibroblast morphology while the RAW264.7 cell is a murine macrophage tumor-derived line. Almost all of the amides were essentially ineffective as inhibitors of cell proliferation when tested at a single 10 μM concentration. One exception was γ-linolenamide that reduced WI-38 cell proliferation by 67%. A previously published report stated that oleamide suppressed the proliferation of human breast carcinoma EFM-18 cells.[32] However, generally speaking the fatty acid amides do not appear to be a promising source of novel anti cancer agents.

A similar series of ethanolamine derivatives (Fig. 1b) was studied under the same conditions as the simple amine derivatives described above. As the data show (Table 2), the inhibitory effects were variable with no clear pattern emerging. The greatest inhibitory action was found with *N*-docosahexaenoyl ethanolamide, which reduced RAW cell proliferation by 64%. The most widely studied compound in this group is *N*-arachidonoyl ethanolamide otherwise known as anandamide, one of the important endocannabinoids. A wide range of actions has been ascribed to anandamide, including anti proliferative activity,[32–34] so it is surprising that it showed little effect in our model. This finding would also tend to be inconsistent with any FAAH inhibition mechanisms for the anti proliferative actions of the acylamido analogs. Another common ethanolamide, *N*-palmitoyl ethanolamide, was not effective in our models, however, others have reported that it is effective in reducing proliferation in human breast cancer cells[34] probably by some indirect mechanism.

Several of the acyl dopamine derivatives whose actions in HeLa cells are shown in Figure 4 were also tested in the WI-38 and RAW cell lines and the results are given in Table 3. In each example, they showed appreciable activity especially in the RAW cell line where every analog produced 90% or better inhibition. Four of these molecules were also studied for possible selectivity of action in the matched HTB cell line and the findings are in Figure 3. These combined results point to the *N*-acyl dopamides as promising template structures for the discovery of novel and safe anti cancer drug candidates. It is worth mentioning that some of these compounds are potent activators of the vanilloid receptor TRPV1,[35] however, an exception is *N*-palmitoyl dopamine that does not activate this receptor. In our studies, *N*-palmitoyl dopamine is both selective and efficacious in reducing cancer cell proliferation suggesting that the TRPV1 receptor is not a mediator for the inhibition of cell proliferation by the acylamido analogs studied here.

Lastly, in Table 4, the question of saturated versus polyunsaturated fatty acids is addressed by directly comparing four classes of palmitoyl and arachidonoyl derivatives in a rat basophilic leukemia line (RBL-2H3). The findings with the glycine and GABA analogs might suggest that saturated fatty acid derivatives are inactive. However, in the L-alanine and dopamine groups both types of fatty acid analogs produced roughly equal degrees of inhibition. Thus, the nature of an SAR for the acylamido analogs remains unclear and the only conclusion at this point is that activity is dependent on both the fatty acid and amino containing components of the molecule.

5. Conclusions

Future studies with the types of fatty acid amido compounds reported on here should be aimed at three important questions. First, the mechanism(s) responsible for the specificity of the anti proliferative actions should be addressed. Second, a more detailed SAR should be obtained that would allow the design of more potent selective analogs. And, finally, the in vivo potential of the more promising compounds should be studied with a view toward their becoming drug candidates. The data presented here justifies carrying out such studies.

6. Materials and methods

6.1.1. Materials

All of the acylamido analogs studied were obtained from BIOMOL International, L.P., Plymouth Meeting, PA 19462 (Screen-WellTM Endocannabinoid Library). The only exception was *N*-palmitoyl tyrosine that was synthesized using a published procedure.[8] All other chemicals were purchased from Sigma-Aldrich, St. Louis, MO 63178. All cell lines were obtained from American Type Culture Collection (ATCC), Manassas, VA 20108. Cell proliferation assay reagents were purchased from Promega Corporation, Madison, WI 53711. Cell culture supplies were obtained from Gibco, Carlsbad, CA.

6.1.2. *N*-arachidonoyl-L-tyrosine; L-EMA-10 (16:0)

Palmitic acid (500 mg) was dissolved in acetonitrile (5 ml) that contained triethylamine (1 ml) and cooled in ice. Isobutylchloroformate (0.5 ml) was then added and the reaction allowed to proceed for 30 min in the ice bath. The reaction mixture was then partitioned between water and ethyl acetate, the organic phase dried over sodium sulfate and evaporated under vacuum. L-tyrosine methyl ester (400 mg) in triethylamine (1 ml)/dimethylformamide (5 ml) was added to the residue and the reaction allowed to proceed for 18 h at 0 °C. The mixture was again partitioned between water and ethyl acetate, washed with 1 N potassium carbonate and then washed with 1 N HCl. The crude methyl ester (190 mg) was then dissolved in a mixture of tetrahydrofuran (5 ml) and 1N LiOH (2 ml) and stirred at room temperature for 18 h. The mixture was acidified with 1 N HCl, dried and evaporated to an oily residue. Trituration with hexane gave a waxy white material (130 mg). ^{1}H NMR (400 MHz $CDCl_3$) 6.94 (d, J = 8.0 Hz, 2H), 6.88 (d, J = 8.0Hz1H), 6.68 (d, J = 8.0 Hz, 2H), 5.81 (m, 1H), 4.55 (m, 1H), 3.10–3.00 (m, 2H), 2.12 (m, 2H), 1.51 (m, 2H), 1.18 (m, 24H) and 0.81 (t, J = 8.0 Hz, 3H). HRMS calcd $C_{25}H_{42}N_1O_4$ (M+H); 420.3114, found; 420.3119 (Δ mass +0.0005).

6.2. Proliferation assay

Cell numbers were estimated using a commercially available kit (Promega). The CellTiter-Glo™ Luminescent Cell Viability Assay kit used here is based on the measurement of ATP, which signals the

presence of metabolically active cells. The assay uses luciferase as the detection enzyme because of the absence of endogenous luciferase activity in mammalian cells. An equal volume of CellTiter-Glo™ Reagent is added to the cell culture and, after 15 min, luminescence is measured. The light signal is proportional to the amount of ATP present, which correlates with the number of viable cells present. The Veritas™ Microplate Luminometer used in this study detects as little as 1.5×10^{-15} moles ATP and values are linear from 760 fg to 5.1 ng of ATP. A study was done to determine the relationship between the experimentally obtained fluorescence response and the actual cell numbers by counting the HTB-125 and HTB-126 cell lines and comparing the assay values (data not shown). In each, the relationship is linear over a wide range; however, the intensity of the luminescence is different for each cell type.

6.3. Cell culture

The culturing conditions are generally those suggested by the supplier, ATTC; however, a brief description for each cell type is given below.

HTB-126™ (Hs 578T). The Hs 578T cell strain was derived from a carcinoma of the breast. It was originated by Hackett et al.[36] along with the Hs 578Bst (see HTB-125 below), which is a normal fibroblast-like line from the same patient. As with Hs 578Bst, no estrogen receptors or endogenous viruses were detected. The donor was a 74-year-old female Caucasian. The cells were not tumorigenic in immunosuppressed mice but were in semisolid medium. The cells are cultured in Dulbecco's Modified Eagle's Medium complete growth medium; the following components are added to the base medium: 0.01 mg/ml bovine insulin and fetal bovine serum to a final concentration of 10%.

HTB-125™ (Hs 578Bst). This is a diploid human cell line that expresses the epidermal growth factor (EGF) receptor. Hs 578Bst was derived by Hackett et al. from normal breast tissue peripheral to an infiltrating ductal carcinoma which was the source for HTB-126.[36] The base medium for this cell line is ATCC Hybri-Care Medium. To make a complete growth medium, the following components are added to the base medium: 1.5 g/l sodium bicarbonate, 30 ng/ml EGF and fetal bovine serum to a final concentration of 10%.

HeLa. The base medium is Gibco Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum added. Cells are grown in a T-75 flask in 15 ml of medium that is replaced on day four and sub cultured on day seven using trypsin to remove the cells. The sub cultivation ratio is approximately 1:10.

WI-38. The base medium is Gibco Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum added. Cells are grown in a T-75 flask in 15 ml of medium, which is replaced on day four and sub cultured on day seven using trypsin to remove the cells. The sub cultivation ratio is approximately 1:10.

RAW264.7. The base medium is Gibco RPMI with 10% fetal bovine serum added. Cells are grown in a T-75 flask in 15 ml of medium; medium is replaced on day four and sub cultured on day seven. Cells are removed by scraping *without* the aid of trypsin. A sub cultivation ratio of 1:3 to 1:6 was used.

RBL-2H3. The base medium is Eagle's Minimal Essential Medium with Earle's BSS and 2 mM L-glutamine (EMEM). In addition to the base medium, the following constituents are added; 1.0 mM sodium private, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate and 15% heat inactivated fetal bovine serum.

6.4. Statistical analysis

All of the plotting, curve fitting and statistical comparisons of data were done with Prism 4 by GraphPad, Inc. All of the data points shown represent the means of 4–6 replicates and the error bars represent S.E.M. when not indicated otherwise. Statistical comparisons were made using an unpaired, two-tailed *t*-test with 95% confidence limits or by one-way ANOVA.

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