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A new strategy to block tumor growth by inhibiting endocannabinoid inactivation

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ABSTRACT

Endocannabinoid signaling has been shown to be enhanced in several cancer tissues and malignant cells, and studies in cell lines have shown that this up-regulation might serve the purpose of providing transformed cells with a further means to inhibit their proliferation. Here we investigated the effect of inhibitors of endocannabinoid degradation on the growth of rat thyroid tumor xenografts induced in athymic mice. VDM-11, a selective inhibitor of endocannabinoid cellular re-uptake, and arachidonoyl-serotonin (AA-5-HT), a selective blocker of endocannabinoid enzymatic hydrolysis, both inhibited the growth in vivo of tumor xenografts induced by the subcutaneous injection of rat thyroid transformed (KiMol) cells. This effect was accompanied by significantly enhanced endocannabinoid concentrations in the tumors excised at the end of the in vivo experiments. Endocannabinoids, as well as VDM-11 and AA-5-HT, inhibited the growth in vitro of the transformed rat thyroid cells used to induce the tumors in vivo, and their effect was reversed at least in part by the cannabinoid CB_1 receptor antagonist SR141716A. This compound, however, when administered alone, did not enhance, but instead slightly inhibited, the growth of rat thyroid transformed cells both in vitro and in tumor xenografts induced in vivo. These findings indicate that endocannabinoids tonically control tumor growth in vivo by both CB_1-mediated and non-CB_1-mediated mechanisms and that, irrespective of the molecular mechanism of their anti-proliferative action, inhibitors of their inactivation might be used for the development of novel anti-cancer drugs.

Key words: AA-5-HT • VDM-11 • KiMol

Stimulation of the membrane receptors specific for the psychoactive principle of *Cannabis*, Δ^9-tetrahydrocannabinol, and for the endogenous cannabinoids, anandamide and 2-archidonoylglycerol (2-AG) (1–3), was previously shown to stop cancer cell proliferation and/or to induce cancer cell apoptosis both in vitro and in vivo (see refs 4, 5 for reviews). In particular, we have shown that the growth of tumor xenografts induced by injecting K*ras*-

transformed rat thyroid cells in athymic mice can be blocked by agents selectively stimulating the cannabinoid CB_1 receptor subtype (6, 7). This effect is due to an anti-mitogenic action caused, at least in part, by inhibition of the activity of the *ras* oncogene product $p21^{ras}$ and by the subsequent *1*) stimulation of the cyclin-dependent kinase inhibitor p27(Kip1) and *2*) inhibition of VEGF signaling and, hence, of neo-angiogenesis. In a separate recent study, we have shown that endocannabinoids inhibit the proliferation also of human colorectal carcinoma cells (8). Endocannabinoid signaling, and/or responsiveness to CB_1 receptor agonists, was increased proportionally to the degree of malignancy in these as well as in other neoplastic cells and tissues, including rat thyroid ras-trasformed cells (6–8). We hypothesized that the endocannabinoid system might be up-regulated in malignant cells in an attempt to reduce cell proliferation and provided preliminary evidence to this hypothesis in CaCo-2 cells in culture (8). In particular, we have shown that treatment of these cells with synthetic substances able to inhibit selectively the inactivation of anandamide and 2-AG, and to increase concomitantly their levels in the cell, leads to inhibition of cell proliferation in vitro in a way that was blocked by a CB_1 receptor antagonist (8). However, no conclusive evidence for a tonic control of cancer growth by the endocannabinoid system has ever been reported.

In the present study, to investigate the possibility that endocannabinoids tonically control tumor cell proliferation in vivo, we have employed the same model of tumor growth that has been used in recent studies to investigate the effect of cannabinoid receptor stimulation on tumor growth, angiogenesis, and metastasis (6, 7). This consists of tumor grafts originated in athymic mice from the injection of K*ras*-transformed rat thyroid cells. We have studied here the effect on the growth of these tumors of several drugs that selectively manipulate the tone of cannabinoid CB_1 receptors by decreasing the degradation of the endocannabinoids, and hence, by increasing their lifespan. We report evidence suggesting that such substances might be used in the future as templates for the development of novel anti-tumor agents.

MATERIALS AND METHODS

Drugs

2-Methyl-2′-F-anandamide (Met-F-AEA) and 2-AG were purchased from Calbiochem. SR141716A was kindly donated by Sanofi-Synthelabo (Montpellier, France). Arvanil, VDM-11, and arachidonoyl-serotonin (AA-5-HT) were synthesized in our laboratory as described previously (9, 10).

Cells and culture

v-K-ras-transformed FRTL-5 cells (KiMol cells) were derived from FRTL-5 cells by infection with a wild-type strain of KiMSV-MolMuLV. We grew KiMol cells at 37°C in Coon's modified Ham's F-12 medium, supplemented with 5% fetal calf serum.

Tumorigenicity assay

All experiments were performed in 6-wk-old male athymic mice (Charles-River). Untreated KiMol cells (1×10^6) were injected subcutaneously into the right flank of 200 athymic mice, and 3 days later animals were randomized and divided in groups of 20 mice each. Met-F-AEA (0.5 mg/kg/dose) or 2-AG at a dose of 5 mg/kg were also injected in the same inoculation region. Re-

uptake inhibitor VDM-11 at a dose of 5 mg/kg, the selective anandamide hydrolysis inhibitor arachidonoyl-serotonin (AA-5-HT) at a dose of 5 mg/kg, and arvanil, a mixed inhibitor of endocannabinoid cellular re-uptake and a partial CB_1 agonist, at a 1.0 mg/kg dose, were likewise injected subcutaneously. SR141716A (0.7 mg/kg) was also administered in the same way. Three different control groups were analyzed where only the vehicle was injected, two groups with the vehicle used in the experiments with Met-F-AEA, 2-AG, VDM-11, AA-5-HT, and arvanil (0.05% ethanol in saline) and one with the vehicle used with SR141716A [0.05% dimethylsulphoxide (DMSO) in saline]. Tumor size was evaluated 20 days after the inoculation. Tumor diameters were measured with calipers every other day until the animals were killed. Tumor volumes (V) were calculated by the formula of rotational ellipsoid: V = A x B2/2 (A=axial diameter, B=rotational diameter). No mouse showed signs of wasting or other visible indications of toxicity. All mice were maintained at the Dipartimento di Biologia e Patologia Animal Facility. Animal experimentations described in the present paper have been conducted in accordance with accepted standards of animal care and in accordance with the Italian regulations for the welfare of animals used in studies of experimental neoplasia, and the study was approved by our institutional committee on animal care.

Cell proliferation assay

Cell proliferation assays were carried out in six-well dishes containing subconfluent cells at a density of 30,000 cells/well and according to the method described previously (6, 7). Several drugs at different concentrations or vehicle (0.5% DMSO) were added for 24 h after which cells were tripsinized and counted by a hemocytometer. Cell viability was assessed with trypan blue with the highest dose tested of the compounds (25 μM).

Measurement of endocannabinoid levels

On the last day of the 5 wk in vivo experiments, tumors (~500 mg wet weight/data point) were excised from either vehicle-treated or drug-treated animals and dounce-homogenized with chloroform/methanol/Tris-HCl 50 mM, pH 7.4 (1/1/1 by vol), containing 100 pmol of d_8-anandamide, 100 pmol of d_4-palmitoyl-ethanolamide, and 200 pmol of d_5-2-AG (Cayman Chemicals) as internal standards. The lipid-containing organic phase was dried down, weighed and prepurified by open-bed chromatography on silica gel (11), and analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) using a Shimadzu HPLC apparatus (model LC-10ADVP) coupled to a Shimadzu (model LCMS-2010) quadrupole MS via a Shimadzu atmospheric pressure chemical ionization (APCI) interface. MS analyses were carried out in the selected ion monitoring (SIM) mode as described previously (12). Temperature of the APCI source was 400°C; HPLC column was a Phenomenex (5 μm, 150x4.5 mm) reverse phase column, eluted as described previously (12). Anandamide (retention time: 14.5 min) and 2-AG (retention time: 17.0 min) quasimolecular ions were quantified by isotope dilution with the above mentioned deuterated standards and their amounts in picomoles nanomoles normalized per milligram of lipid extract.

RESULTS AND DISCUSSION

The rationale behind this study was provided by a simple consideration based on previous studies: if endocannabinoid levels are enhanced in some tumors (8, 13, 14), and if these substances can exert a tonic inhibition of cancer cell proliferation, as suggested by in vitro

experiments carried out with human colorectal cancer cells (8), then substances that inhibit the ongoing degradation of endocannabinoids also should be capable of inhibiting cancer growth in vivo. This possibility would open the way to new anti-cancer drugs that should be devoid of, or at least limit, the possible psychotropic or immunosuppressive side effects typical of "direct" agonists of cannabinoid receptors (4, 5), since these substances would lead to activation of cannabinoid receptors preferentially where an ongoing synthesis and degradation of endocannabinoids occurs, i.e., at the site of cancer growth. To test this hypothesis, we had to demonstrate, first, that selective inhibitors of endocannabinoid degradation can inhibit cancer growth in vivo as efficaciously as cannabinoid receptor agonists and, second, that these substances were indeed acting by prolonging endocannabinoid lifespan in the tumors.

Substances were coadministered the first time together with the subcutaneous inoculation of the K*ras*-transformed thyroid cells into athymic mice and then intratumor twice a week for the 5 wk duration of the experiment. Mice that had been treated with vehicle alone developed subcutaneous tumors the size of which was around 5-6 mm^3 after 5 wk. In agreement with previous studies (6), administration of the metabolically stable anandamide analog Met-F-AEA (0.5 mg/kg/dose) significantly inhibited the growth of the tumors and their size at the end of the experiment (Fig. 1*A*, *B*). We also showed here for the first time that administration of the other endocannabinoid 2-AG, at a dose higher than that of Met-F-AEA (5 mg/kg), causes a smaller, albeit still statistically significant ($P<0.05$), reduction of tumor size (Fig. 1*A*). The fact that this compound is less efficacious, based on the single dose injected, than Met-F-AEA is hardly surprising if one takes into consideration that 2-AG was shown to undergo degradation in vivo more rapidly than anandamide (15). Most importantly, intratumor administration of the selective endocannabinoid re-uptake inhibitor VDM-11 and of the selective anandamide hydrolysis inhibitor AA-5-HT, both at the dose of 5 mg/kg, strongly and significantly inhibited tumor growth over the course of the experiments (Fig. 1*B*). At the end of the experiments, the average size of the tumors treated with VDM-11 and AA-5-HT was ~37 and ~75% of those treated with vehicle. The two inhibitors were selected mostly because of their proven selectivity. VDM-11 is an inhibitor of the putative endocannabinoid membrane transporter, and as such it inhibits anandamide cellular uptake (IC_{50}=8-10 μM) but, unlike other similar inhibitors, has little effect on both anandamide hydrolysis and vanilloid TRPV1 receptors (10). AA-5-HT is a metabolically stable inhibitor of fatty acid amide hydrolase (FAAH), the enzyme mostly responsible for anandamide hydrolysis (16), which under certain conditions can also catalyze 2-AG hydrolysis (17). This compound exhibits, against FAAH, IC_{50} values between 6 and 12 μM but, unlike some other inhibitors of its kind, was tested also against other proteins that have been found to be inhibited by other FAAH inhibitors, such as phospholipases A_2, and found to be inactive (9). Since both compounds have almost no affinity for cannabinoid CB_1 or CB_2 receptors (K_i>10 μM), and their potency on the respective targets is very similar, their different efficacy on tumor xenografts growth, observed here, might be due to their different pharmacokinetics, as suggested by their different potencies in vitro (see below), or they may reflect a different relative weight of the uptake and hydrolysis mechanisms upon endocannabinoid degradation. At any rate, it is important to underline that no inhibitory effect of either VDM-11 or AA-5-HT on locomotion was observed during the course of the experiments (data not shown), in agreement with previous data showing that these two compounds are inactive on this behavioral parameter of central cannabimimetic activity even when administered systemically (9, 18).

Interestingly, arvanil, a metabolically stable, mixed inhibitor of endocannabinoid cellular re-uptake and partial CB_1 agonist, which is inactive on FAAH, also significantly inhibited tumor

growth (down to ~60% of vehicle treated tumors at a 1.0 mg/kg dose, intratumor; Fig. 1*A*). This compound is a cannabinoid CB_1 receptor agonist (K_i=0.5-1.9 μM; ref 19) and an inhibitor of endocannabinoid re-uptake (IC_{50}=3.5 μM; ref 10). Furthermore, this compound is a very potent (K_i=0.28 μM, EC_{50} ~0.5 nM) agonist of vanilloid receptors (10, 19), which can also be directly activated by anandamide (20) and have been implicated in inhibition of cancer cell growth through induction of apoptosis (21–23). The extent of the effect of arvanil as compared with VDM-11, which is chemically very similar to arvanil and hence is likely to have a similar pharmacokinetic profile, suggests that *1*) vanilloid receptors and induction of apoptosis are not involved in the anti-cancer effects of either endocannabinoids or of inhibitors of their inactivation, at least in the in vivo model used in this study (see below); and *2*) no further enhancement of the cancer growth-inhibitory actions of a compound is achieved when the capability to directly activate CB_1 receptors is added to its ability to activate this receptor indirectly via inhibition of endocannabinoid re-uptake. However, a full assessment of the actual involvement of vanilloid receptors in the assay of tumorigenicity used here, as well as in possible vascular effects implicated in tumor growth, was not among the aims of the present study and should be investigated further by using specific agonists and antagonists for these receptors.

We next needed to demonstrate that VDM-11 and AA-5-HT were indeed acting through the enhancement of endocannabinoid tissue concentrations. Therefore, we compared the levels, as measured by isotope-dilution liquid chromatography mass spectrometry, of anandamide and 2-AG in tumors excised from treated and untreated mice on the last day of the in vivo experiments described above. AA-5-HT produced a significant elevation of the concentrations of both anandamide and 2-AG. The finding of a stimulatory effect by a FAAH blocker also on 2-AG, which has been reported to be predominantly hydrolyzed by a monoacylglycerol lipase and not by FAAH (24, 25), can be regarded as surprising. However, FAAH inhibition by AA-5-HT has been already shown to elevate 2-AG levels in isolated cells (8), and it is possible that intratumor, as opposed to systemic (25), administration of FAAH inhibitors unmasks a possible tonic role of FAAH in limiting also 2-AG levels. Again surprisingly, VDM-11 only enhanced 2-AG levels (Fig. 2). This could be due to the fact that the putative endocannabinoid transporter has a higher affinity for anandamide with respect to 2-AG (26) and that, therefore, higher amounts of VDM-11 might be required to also inhibit anandamide uptake in vivo. In agreement with its mechanism of action via inhibition of FAAH, AA-5-HT also elevated the levels of the FAAH substrate, and anandamide anti-inflammatory congener, palmitoylethanolamide (27), although to a lesser extent, possibly because this compound can be hydrolyzed also by another amidase (17). These findings, together with the different efficacy of VDM-11 and AA-5-HT (Fig. 1), suggest that elevation of endogenous 2-AG levels might be sufficient alone to inhibit tumor xenografts growth "from inside," possibly because this compound is 150- to 300-fold more abundant than anandamide. These data indicate that tumor growth inhibition in vivo can be achieved, with little if any psychotropic effect, by using agents that act by enhancing tumor endocannabinoid levels. Also, the observation that VDM-11, which enhanced significantly only 2-AG levels, was more efficacious than 2-AG at blocking tumor growth, albeit based solely on the comparison between just one dose of the two compounds, suggests that enhancing the local levels of an endocannabinoid may achieve better results in terms of tumor growth inhibition in vivo than administering exogenously the metabolically unstable compound.

We next investigated the mechanism of action of VDM-11 and AA-5-HT by studying their effects in vitro on the same transformed rat thyroid cells used to induce the tumors in vivo. The two compounds, as well as 2-AG, Met-F-AEA, and arvanil, dose-dependently inhibited cell

proliferation with IC_{50} below 10 μM in all cases, and with no effect on apoptosis (Fig. 3 and data not shown). The CB_1 receptor antagonist SR141716A (0.2 μM, Fig. 3), but not the CB_2 receptor antagonist SR144528 (0.5 μM, data not shown), counteracted significantly the effects of Met-F-AEA and VDM-11, thus suggesting that CB_1 receptors are uniquely involved in the in vitro anti-proliferative effects of these compounds. The effects of 2-AG, AA-5-HT, and arvanil were inhibited by SR141716A only to a little extent, thus suggesting that these compounds might act also through other, non-CB_1-non-CB_2-mediated mechanisms. Importantly, however, SR141716A (Fig. 3), but not SR144528 (not shown), also exerted a significant, albeit small, anti-proliferative action per se. This effect, the mechanism of which was not investigated here, is likely to have masked in part the antagonism of 2-AG, AA-5-HT, and arvanil actions. Indeed, a possible, although not necessary, consequence of endocannabinoids exerting a tumor growth-inhibitory tone by acting through cannabinoid CB_1 receptors would be that blockade of these receptors might cause an enhancement of tumor growth in vivo. However, SR141716A, at a dose (0.7 mg/kg, intratumor, twice a week for 5 wk) sufficient to block Met-F-AEA inhibitory action in the same model (6), exerted a small albeit significant anti-tumor effect (Fig. 4), thus reflecting again the effect observed in vitro. The lack of a pro-proliferative action by SR141716A is, indeed, not surprising as it indicates that, although endocannabinoids, by stimulating CB_1 receptors, might help down-regulating tumor growth in vivo, this represents only one out of many possible tumor-suppressing mechanisms occurring during cell transformation. Furthermore, the finding of small inhibitory effects on cancer cell proliferation by the CB_1 receptor antagonist suggests that endocannabinoids, and 2-AG in particular, might exert their tonic anti-proliferative effects also through non-CB_1 receptor-mediated mechanisms, particularly when CB_1 receptors are blocked. These mechanisms are not likely to involve cannabinoid CB_2 receptors, because we have previously shown that these receptors are not involved in endocannabinoid anti-proliferative effects (6–8), and because we have found here that the CB_2 antagonist has no effect on the anti-proliferative effects in vitro of the compounds tested. However, apart from vanilloid receptors (see above) several other membrane proteins and ion channels are being proposed as endocannabinoid molecular targets under both physiological and pathological conditions (28). The exact mechanism underlying the small anti-cancer effect of SR141716A will clearly require further investigations.

Irrespective of the mechanism of the anti-proliferative actions of endocannabinoids, and, in fact, in view of the possibility that they may inhibit cancer cell growth and spreading also via noncannabinoid receptor-mediated pathways, our data suggest that an alternative and possibly more efficacious and safer strategy to block tumor growth in vivo may be by using substances that selectively inhibit endocannabinoid degradation. These compounds should be more specific than traditional CB_1 receptor agonists since they are likely to affect endocannabinoid levels, and hence the state of activation of CB_1 receptors, only in those tissues where these compounds are produced "on demand" to contribute exerting protective actions, and not, as in the case of cannabinoid receptor agonists, in all tissues expressing functionally active CB_1 receptors. Many examples of the successful use of endocannabinoid uptake and hydrolysis inhibitors in animal models of other disorders have been reported recently, e.g., for anxiety (25), cholera toxin-induced intestinal hypersecretion (29), kainate-induced excitotoxicity (30), and glutamate hyperactivity of corticostriatal neurons in Parkinson's disease (31). However, to the best of our knowledge, this is the first report that such substances, by strengthening an anti-proliferative tone of endogenous cannabinoid levels, can also inhibit cancer growth in vivo. Only further experiments will tell whether the present findings will result in the development of new anti-cancer drugs against endocannabinoid-sensitive tumors (5).

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Fig. 1

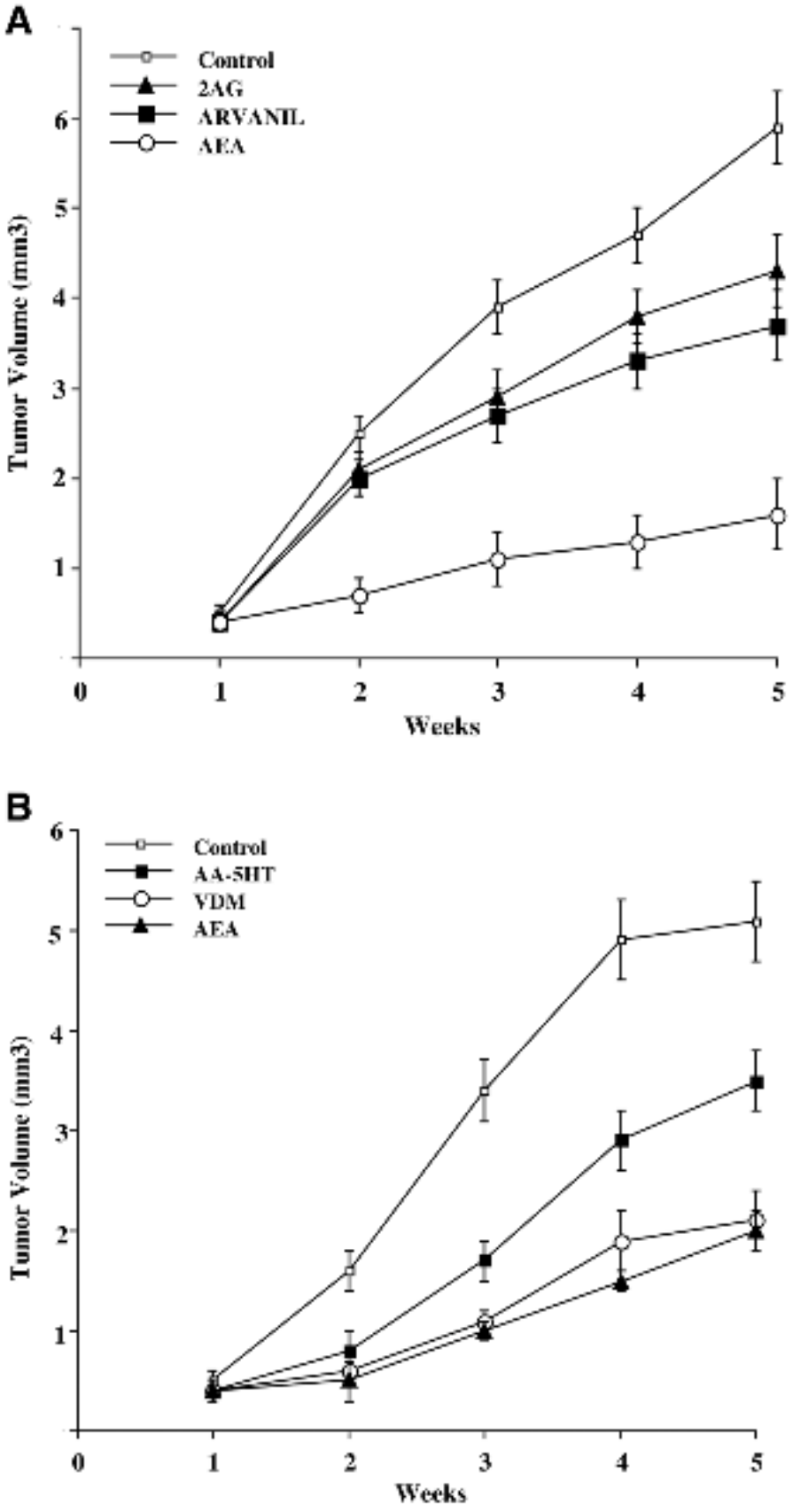
A
Control
2AG
ARVANIL
AEA
Tumor Volume (mm3)
0
1
2
3
4
5
6
Weeks
B
Control
AA-5HT
VDM
AEA
Tumor Volume (mm3)
0
1
2
3
4
5
6
Weeks

Figure 1. ***A*****)** Effect of intratumor administration of 2-methyl-2'-F-anandamide (AEA, 0.5 mg/kg/dose), 2-arachidonoyl-glycerol (2-AG, 5 mg/kg/dose), and arvanil (1 mg/kg/dose) on the growth of tumor xenografts induced in athymic mice by KiMol cells (see Materials and Methods). ***B*****)** Effect of intratumor administration of AEA (0.5 mg/kg/dose), VDM-11 (VDM, 5 mg/kg/dose), and arachidonoyl-serotonin (AA-5-HT, 5 mg/kg/dose) on the growth of tumor xenografts induced in athymic mice by KiMol cells. Data are means ± SE of $n = 10$ mice per data point. Effects of all compounds at all time points were statistically different ($P<0.05$ by ANOVA followed by the Bonferroni's test) from vehicle (control).

Fig. 2

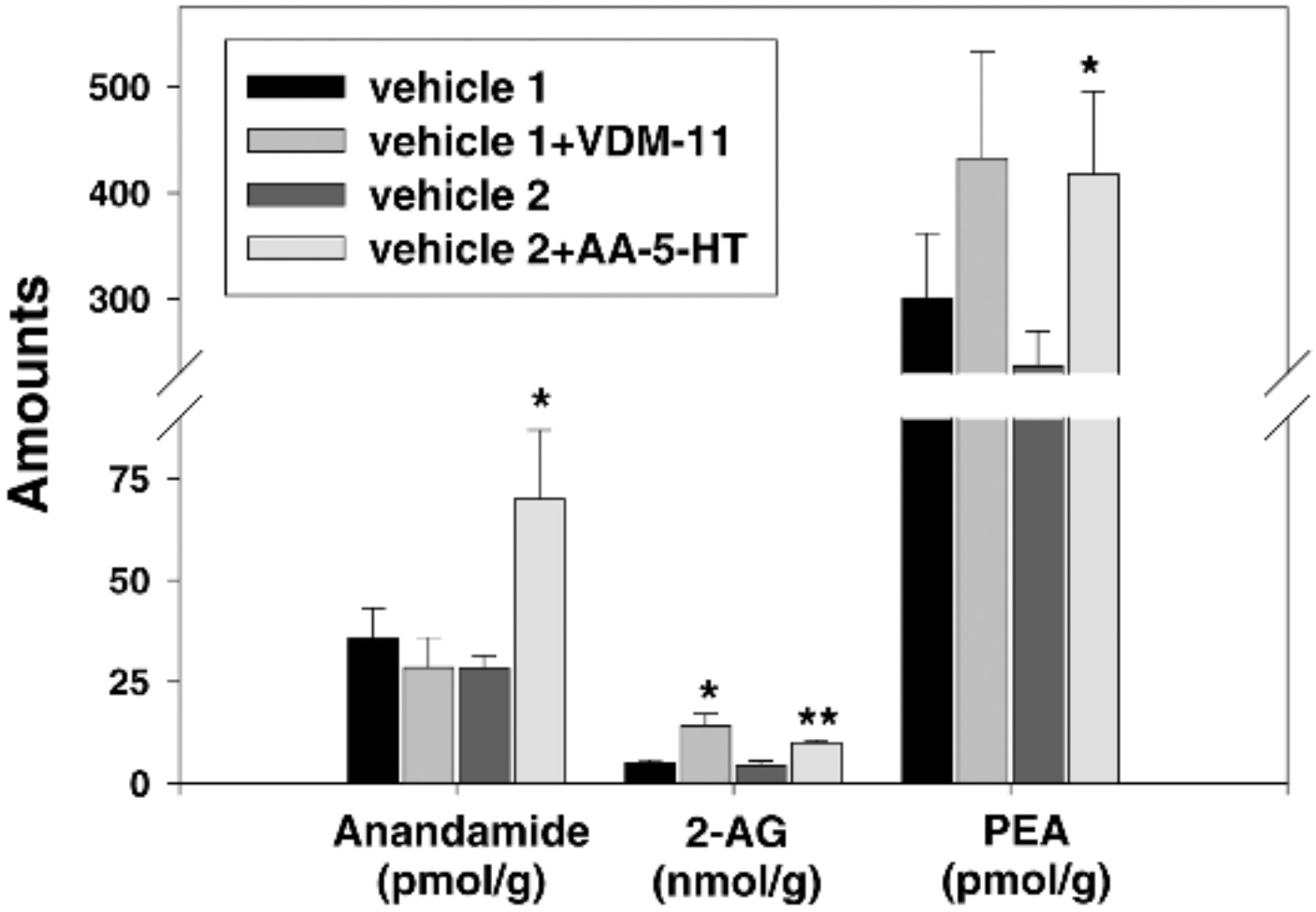

Figure 2. Effect of VDM (5 mg/kg/dose) and AA-5-HT (5 mg/kg/dose) on the levels of anandamide, palmitoylethanolamide (PEA), and 2-AG in tumors excised from athymic mice, after a 5-wk intratumor treatment with the 2 substances or vehicle. Anandamide, PEA, and 2-AG levels were measured by isotope dilution-liquid chromatography-mass spectrometry in tissue lipid extracts as described in the Materials and Methods. Basal tumor levels from 2 sets of vehicle-treated mice are shown. Data are means ± SE of $n = 5$ mice per data point. *$P < 0.05$; **$P < 0.01$ vs. control (vehicle), by ANOVA followed by the Bonferroni's test.

Fig. 3

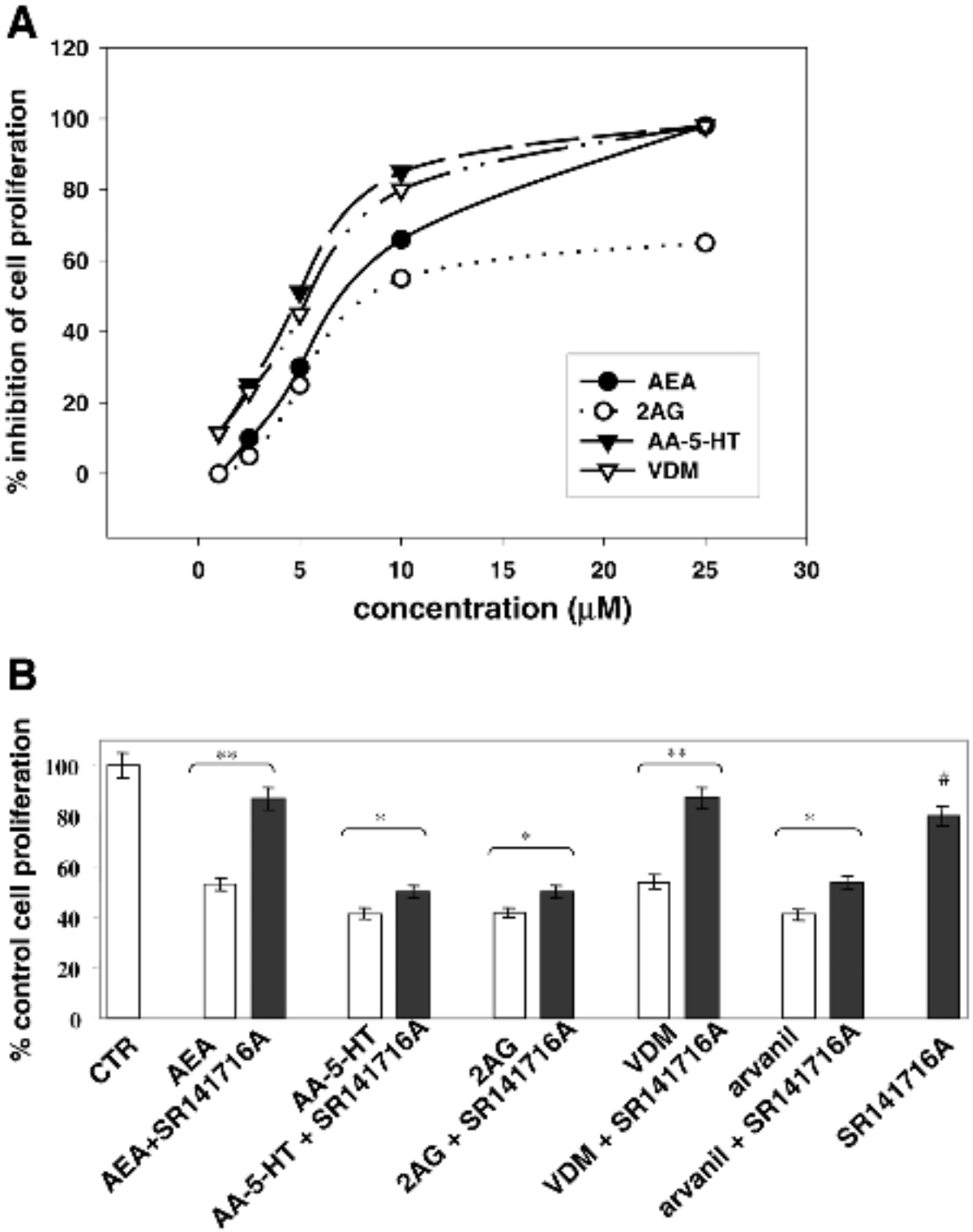

Figure 3. Effect of several substances on the proliferation of K-ras-transformed rat thyroid cells (K_i-Mol cells). Data are means ± SE of n = 10 mice per data point. All compounds produced a statistically significant inhibition of cell proliferation (P<0.05). ***A***) Dose dependency of the effect of AEA, VDM, AA-5-HT, and 2-AG. ***B***) The CB_1 receptor antagonist SR141716A (0.2 μM) counteracted the inhibitory actions of AEA, VDM, AA-5-HT, and arvanil (*P<0.05; **P<0.01 vs. drug only without SR141716A); SR141716A also exerted a small inhibitory effect [#P<0.05 vs. control (CTR)]; by ANOVA followed by the Bonferroni's test. The CB_2 antagonist SR144528 (0.5 μM) was also tested together with AEA, 2-AG, VDM, and AA-5-HT and did not significantly modify the effects of these substances (not shown).

Fig. 4

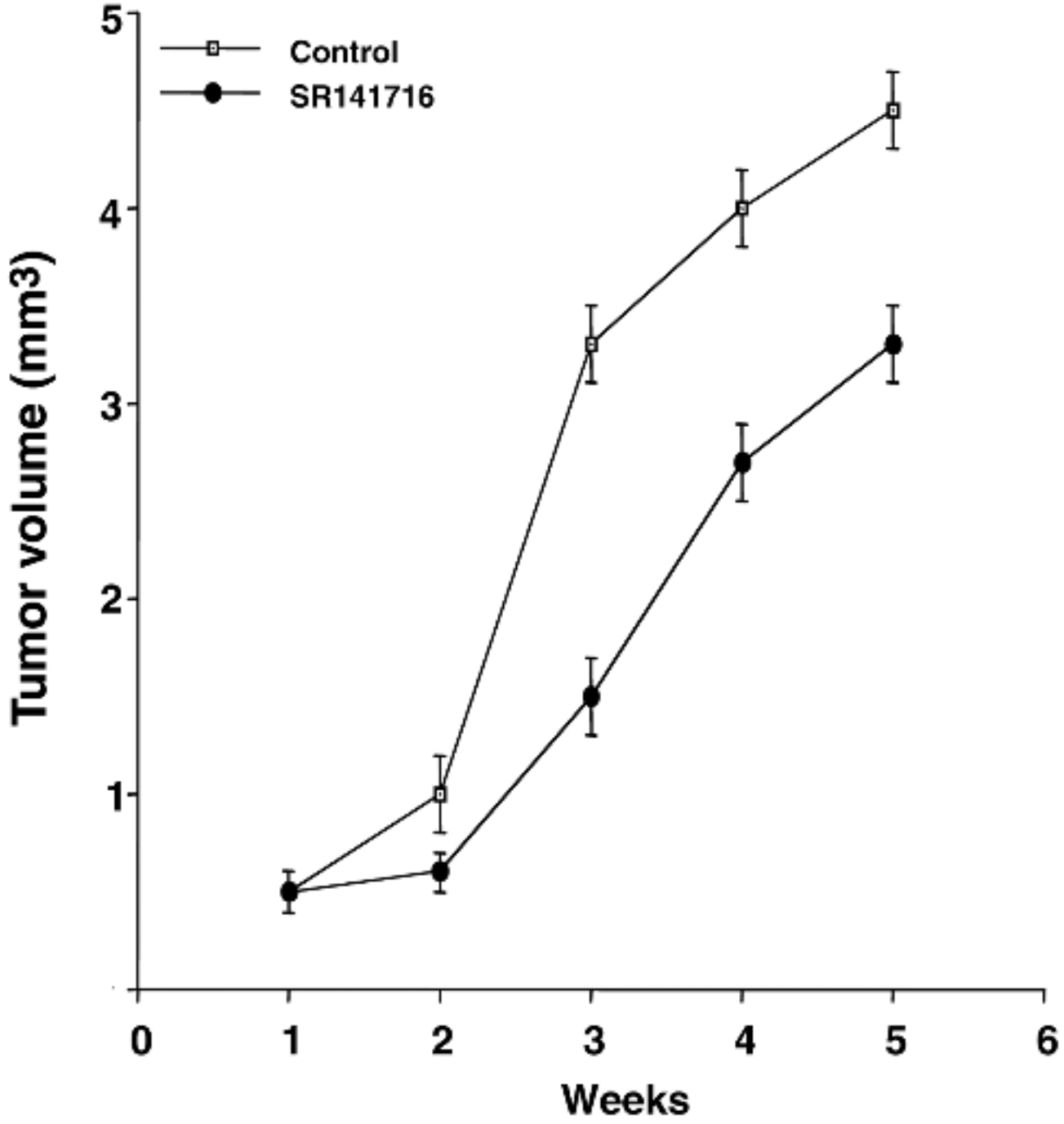

Figure 4. Effect of intra-tumor administration of SR141716A (AEA, 0.7 mg/kg/dose) on the growth of tumor xenografts induced in athymic mice by KiMol cells (see Materials and Methods). Data are means ± SE of $n = 10$ mice per data point. The effects of the compound at all time points were statistically different ($P<0.05$ by ANOVA followed by the Bonferroni's test) from vehicle (control).