Cannabinoid receptor 1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma

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Abstract
Gene expression profiling has revealed that the gene coding for cannabinoid receptor 1 (CB1) is highly up-regulated in rhabdomyosarcoma biopsies bearing the typical chromosomal translocations PAX3/FKHR or PAX7/FKHR. Because cannabinoid receptor agonists are capable of reducing proliferation and inducing apoptosis in diverse cancer cells such as glioma, breast cancer, and melanoma, we evaluated whether CB1 is a potential drug target in rhabdomyosarcoma. Our study shows that treatment with the cannabinoid receptor agonists HU210 and Δ9-tetrahydrocannabinol lowers the viability of translocation-positive rhabdomyosarcoma cells through the induction of apoptosis. This effect relies on inhibition of AKT signaling and induction of the stress-associated transcription factor p8 because small interfering RNA-mediated down-regulation of p8 rescued cell viability upon cannabinoid treatment. Finally, treatment of xenografts with HU210 led to a significant suppression of tumor growth in vivo. These results support the notion that cannabinoid receptor agonists could represent a novel targeted approach for treatment of translocation-positive rhabdomyosarcoma. [Mol Cancer Ther 2009;8(7):1838–45]

Introduction
Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children, representing 5% to 8% of all childhood malignancies (1). It is believed to originate from muscle precursor cells and histology recognizes two major subtypes: The embryonal subtype (eRMS) accounts for ∼60% of RMS cases and has a rather good prognosis (2). The alveolar subtype (2) is less frequent, more aggressive, usually presents with metastasis, and is thus associated with rather poor treatment outcome. Although no consistent genetic alterations have been identified thus far in eRMS, ∼80% of aRMS patients display typical chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14) encoding for fusion proteins PAX3/FKHR or PAX7/FKHR, respectively (3). These chimeric transcription factors are oncogenic and presumably act mainly through their gain in transcriptional activity.

To provide insight into molecular changes elicited by these transcription factors and to find new potential therapeutic targets for treatment of RMS, gene expression analysis was done in a range of RMS biopsies by several research groups (4–6). These studies consistently revealed a gene expression signature of up-regulated genes in translocation-positive versus translocation-negative samples. Interestingly, translocation-negative aRMS clustered together with eRMS samples in these analyses. Hence, at the molecular level, RMS can be divided into translocation-positive RMS (tposRMS) and translocation-negative RMS (tnegRMS).

The gene expression signature of tposRMS contains a number of receptor molecules that might be potentially amenable as drug targets. Among these, receptors such as c-met have already been validated as therapeutic target (7). However, one of the top-ranking genes in this signature is the cannabinoid receptor 1 (CB1). Thus far, no studies have been undertaken to assess whether CB1 might serve as a future target for therapeutic intervention in this tumor. Evidence exists since 1975 that cancer cell growth can be inhibited by treatment with cannabinoid receptor agonists, as first described by Munson et al. in Lewis lung carcinoma cells (8). Since then, additional cancer cell types such as glioblastoma (9), breast carcinoma (10), or melanoma (11) were reported to be sensitive to the antiproliferative action of cannabinoids. In general, the antitumoral actions of diverse cannabinoid receptor agonists are mediated through the cannabinoid receptor types CB1 and CB2, as reviewed by Guzman et al. (12). Notably, not only in vitro cell culture systems are subject to this treatment response but also in vivo experiments using either xenografts or syngeneic mouse models have shown the potential of cannabinoids as anticancer agents, without observing major psychoactive or immune-suppressive effects (8, 13). Recently, the first clinical study using Δ9-tetrahydrocannabinol (THC) in severe cases of glioblastoma has been reported (14).

At the molecular level, cannabinoids trigger changes in various signaling pathways in cancer cells. One of the primary events after cannabinoid treatment is a sustained de novo synthesis of the lipid second messenger ceramide, which in turn is followed by inhibition of AKT signaling (15). Strikingly, both of these signaling events mark a
major difference between tumor cells and healthy non-transformed cells, which undergo AKT activation without de novo synthesis of ceramide after cannabinoid stimulation (16). In parallel to AKT inhibition, alterations in extracellular signal-regulated kinase (ERK) signaling have been reported. However, depending on tumor type, either inhibition (17) or sustained activation (18) has been observed. Recently, the stress-associated transcription factor p8 was found to be critically involved in cannabinoid-induced apoptosis of cancer cells (19) as its down-regulation could rescue viability of various cancer cells (20, 21). At the end of the signaling cascade, tumor cells either undergo cell cycle arrest (22, 23) or apoptosis (9, 24).

To improve the treatment outcome of the aggressive tposRMS subtype, novel targeted therapies are urgently needed. Therefore, this study aimed to characterize the effects of cannabinoids on tposRMS cells in vitro as well as in vivo. Our results show that the CB1 receptor could represent a potential molecular target for future therapeutic approaches in tposRMS.

Materials and Methods

Cannabinoids

HU210 ([(+)-1,1-dimethylheptyl analogue of 11-hydroxy-D8-tetrahydrocannabinol] was purchased from Tocris, 2-methyl-2′-F-anandamide (Met-F-AEA) from Cayman, AM251 (analogue of SR141716A) from Sigma-Aldrich, and THC from The Health Concept. All substances were solved in DMSO. For in vitro experiments, they were applied at final DMSO concentrations of maximally 0.05% (v/v). For in vivo experiments, HU210 was prepared at 0.25% DMSO (v/v) and diluted in PBS supplemented with 5 mg/mL bovine serum albumin.

Cell Culture

Rh4 and Rh28 tposRMS cells were kindly provided by P. Houghton (St Jude’s Children’s Research Hospital, Memphis, TN, USA); RMS13, RD, and MRC-5 lung fibroblast cells were obtained from the American Type Culture Collection (LGC Promochem). All cells were routinely maintained in DMEM supplemented with 10% FCS. When performing viability or signaling experiments, cells were plated at a density of 17,000/cm², allowed to adhere overnight, and transferred to serum-free medium 6 h before starting drug treatments.

Cell Viability and Apoptosis Detection

Cell viability was evaluated in 96-well plates using methylthiazolyldiphenyl-tetrazolium bromide from Sigma-Aldrich. Apoptosis was either analyzed with CospGLOW red active caspase-3 staining kit (Biovision), allowing labeling of apoptotic cells with a fluorescent caspase-3 substrate and subsequent detection by fluorescence microscopy. Alternatively, the apoptosis-indicating ratio of cleaved to uncleaved poly(ADP-ribose)polymerase (PARP) protein was determined densitometrically by Western blotting.

Reverse Transcriptase-PCR

RNA was extracted with the RNeasy Mini Kit (Qiagen), including a DNase digestion step with RNase-free DNase (Qiagen). One microgram of total RNA was reverse transcribed with random hexamer primers using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). PCR was done with primers for hCB1 (5′-CGGCGCAAGGCTGACT-3′ and 5′-CATGCCGCTTGGCTCAG-3′) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Microsynth) using the following parameters: After initial denaturation at 94°C for 5 min, cycles (40×) with 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s was done, followed by a final extension at 72°C for 5 min.

Quantitative Real-time PCR

Quantitative reverse transcription-PCR (RT-PCR) was done under universal cycling parameters on an ABI7900HT instrument using commercially available Mastermix and target probes for CB1, p8, and GAPDH (all from Applied Biosystems). Cycle threshold (Ct) values were normalized to GAPDH. Relative expression levels of the target genes among the different samples were calculated using the ΔΔCt method.

Gene Silencing

Rh4 cells were transfected with 10 nmol/L small interfering RNA (siRNA) against human p8 (Qiagen) or scrambled siRNA (Ambion) using GeneEraser (Stratagene). One day after siRNA transfection, equal numbers of cells were plated for subsequent viability experiments. p8 down-regulation efficiency was verified by means of quantitative RT-PCR.

Western Blot Analysis

For detection of intracellular signaling proteins, whole-cell extract was produced with a lysis buffer consisting of 50 mmol/L Tris (pH 7.5), 1% Triton X-100, 1 mmol/L EGTA, 50 mmol/L NaF, 10 mmol/L sodium β-glycerophosphate, 5 mmol/L sodium PPI, 1 mmol/L sodium orthovanadate, 1% phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, and protease inhibitor cocktail complete (Roche) according to standard protocols. Samples were sonicated and equal amounts of protein were used for Western blotting with the NuPAGE system (Invitrogen). Antibodies used for detection included rabbit antibodies raised against CB1 (1:1,000; Affinity Bioreagents), PARP, phospho-AKT (Ser473), phospho-AKT (Thr308), AKT-total, phospho-ERK (Thr202/Tyr204), ERK-total, phospho-GSK (Ser9/21), and GSK (all 1:1,000; from Cell Signaling Technology). Detection of actin with a rabbit antibody (1:2,000; Sigma-Aldrich) was used to control for equal protein loading. As secondary antibody, an anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Pierce) was used. Detection was done with ECL technology (Amersham).

Confocal Microscopy

Cells on coverslips were fixed with paraformaldehyde (PFA) and incubated with anti-CB1 antibody (1:500; Affinity Bioreagents) in PBS/2.5% goat serum for 0.5 hours at 37°C. For visualization, an anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Pierce) was used. Detection was done with ECL technology (Amersham).
Immunohistochemical Staining
Tumors were fixed, embedded in paraffin, and sectioned into 2-μm slices. Immunohistochemical stainings for Ki67 (Lab Vision Corporation) and cleaved caspase-3 (Cell Signaling Technology) were done on the Ventana Benchmark automated staining system (Ventana Medical Systems).

Tumorigenicity Assay
Rh4 cells (7.5 × 10^6) in 100 μL PBS were injected s.c. into the flank of NOD/LtSz-scid IL2Rγ null (NOG) mice (The Jackson Laboratory). When tumors reached a size of 150 mm^3, mice were randomly assigned to treatment and control groups and injected peritumorally for 13 d with 0.2 mg/kg HU210 or vehicle (DMSO) alone. Tumor growth was monitored daily with external caliper, and the tumor volume was calculated as (4/3π((width + length)/4)^3. Animals were sacrificed 1 d after the last treatment.

Statistical Analysis
Statistical analysis was done with two-tailed t test with the statistics program SPSS. For analysis of tumor growth, a longitudinal analysis was done by comparing linear regressions of the two groups.

Results
The CB1 Receptor Is Up-Regulated in Translocation-Positive RMS
Gene expression profiling of RMS biopsy samples shows a signature for tposRMS (4) that includes, as one of the top-ranking genes, CNR1, encoding the CB1 receptor (Fig. 1A). In contrast, transcript levels of the related CNR2 gene, encoding the CB2 receptor, were only slightly above background. To validate the up-regulation of CB1 in tposRMS cells on both the RNA and protein levels, we first applied conventional RT-PCR, which revealed a higher expression of CB1 mRNA in all tposRMS cells than in control cell lines MRC-5 (fibroblast) and RD (eRMS; Fig. 1B). Indeed, expression was >10,000-fold higher than in the controls when assessed quantitatively. In addition, expression of CB1 is 430-fold higher than expression of CB2 in Rh4 cells, whereas in U87MG glioma and A375 melanoma cells, expression of CB2 is more prevalent (data not shown). Further, CB1 was expressed in Rh4 cells also at the protein level as shown by Western blot (Fig. 1C). Last, confocal microscopy of cultured Rh4 and RD cells (Fig. 1D) showed higher immunofluorescence staining intensities for CB1 in Rh4 cells. Hence, expression of CB1 is evident both on the mRNA and protein levels in tposRMS cells, confirming the previous findings using gene expression profiling.

Cannabinoids Reduce the Viability of tposRMS Cells
In vitro
After validating the expression of CB1 in tposRMS cells, we next assessed the cell viability after treatment with different cannabinoid receptor agonists. Treatment with the mixed cannabinoid receptor agonist HU-210 reduced the viability of two tposRMS cell lines in a dose-dependent manner but not of the tnegRMS cells (RD) or control nontransformed fibroblasts (MRC-5), which express lower levels of the CB1 receptor (Fig. 2A and B). Finally, pharmacologic blockade of the CB1 receptor significantly restored cell viability of Rh4 cells from 29.2% (±2.45SD) to 71.9% (±11.2 SD; Fig. 2B), supporting the notion that the observed reduction in cell viability was specifically mediated through the CB1 receptor.

Cannabinoids Induce Apoptosis in tposRMS Cells
To determine whether decreased cell viability in tposRMS cells after cannabinoid treatment is due to apoptosis, caspase-3 activation and PARP cleavage were analyzed. First, tposRMS cells were treated with 1.25 μmol/L HU210 and cell extracts...
were analyzed by immunoblotting for PARP cleavage at different time points (Fig. 3A). Already 6 hours after start of treatment, PARP cleavage could be observed and after 24 hours, almost no uncleaved protein was detectable. Treatment of Rh4 and Rh28 cells with increasing HU210 concentrations showed a similar increase in PARP cleavage, whereas pretreatment of Rh4 cells with 0.5 μmol/L of the CB1-specific antagonist AM251 significantly rescued cleavage of PARP protein (Fig. 3B). At a concentration of 1.25 μmol/L HU210, for example, the ratio of cleaved to uncleaved PARP protein could be rescued from 1.13 (±0.05 SD) to 0.40 (±0.03 SD; Fig. 3C).

Additionally, caspase-3 activation after 24 hours of HU210 treatment was measured in Rh4 cells (Fig. 3D). A concentration-dependent increase of cells positively stained for active caspase-3 was detected with close to 100% apoptotic cells at 1.25 μmol/L. In line with the previous results, we also observed that both THC (2 and 4 μmol/L) and Met-F-AEA (5 and 10 μmol/L) treatment induced cleavage of PARP protein after 24 hours of incubation (Fig. 3E and F). Therefore, treatment of tposRMS with cannabinoids induces apoptosis in tposRMS cells.

Cannabinoids Inhibit AKT Signaling

Earlier studies investigating effects of cannabinoids on cancer cells could show alterations in AKT and ERK signaling upon drug treatment. Based on this, we next studied AKT and ERK signaling in tposRMS cells after cannabinoid treatment. Among the tposRMS cell lines, Rh4 cells most accurately reflect the translocation-specific gene expression signature and therefore this cell line was selected as model system for further studies. They were incubated with 1.25 μmol/L HU210 for 30 minutes and 2 hours before cell lysis. A rapid decrease in phospho-AKT at Ser473 was detected, indicating inhibition of AKT activity (Fig. 4A). Under the same experimental conditions, phosphorylation of ERK was found to increase in tposRMS cells after drug treatment compared with vehicle-treated cells, at both Thr202/Tyr204 (Fig. 4A, bottom left). Further phosphorylation on Thr308 of AKT was also reduced. In addition, the AKT downstream target GSK3β became significantly dephosphorylated (Fig. 4A, right). Notably, also THC (2 and 4 μmol/L) as well as Met-F-AEA (5 and 10 μmol/L) triggered dephosphorylation of AKT at Ser473 (Fig. 4B) with a slight delay compared with HU210. No difference for ERK phosphorylation was observed with either of these two substances (data not shown). In summary, all three cannabinoid agonists lead to inhibition of AKT signaling in tposRMS cells, whereas ERK activation was only seen after treatment with HU210. These experiments suggest that the AKT pathway is likely to mediate the action of cannabinoids in our tumor model.

Cannabinoids Reduce Viability through Up-Regulation of Transcription Factor p8

p8 is a transcription factor involved in cellular stress responses following cellular injuries through pathways implicated in growth inhibition (25, 26). Furthermore, p8 mediates apoptosis upon cannabinoid treatment of glioblastoma (19), pancreatic cancer (20), and breast cancer (21) cells. Therefore, we tested involvement of p8 in tposRMS cells, whereas ERK activation was only seen after treatment with HU210. These experiments suggest that the AKT pathway is likely to mediate the action of cannabinoids in our tumor model.

Figure 2. Cannabinoids reduce viability of tposRMS cells. A, cell lines Rh4, Rh28 (tposRMS), RD (tnegRMS), and MRC-5 (fibroblasts) were incubated with increasing concentrations of HU210 for 72 h. Subsequent viability measurements by means of MTT are shown (n = 3, ±SE; significance at 1.25 μmol/L; P < 0.005); B, viability measurements are shown for Rh4 cells preincubated with vehicle or with 0.5 μmol/L AM251 before undergoing subsequent treatment with 1 μmol/L HU210 for 24 h (n = 3, ±SE; significance: P < 0.05); C and D, dose-dependent viability of Rh4, RD, and MRC-5 cells after treatment with THC for 24 h or Met-F-AEA for 48 h was measured (n = 3, ±SE, significance at 5 μmol/L THC and 10 μmol/L Met-F-AEA: P < 0.05).
To validate the requirement of p8 up-regulation for induction of apoptosis, p8 expression was specifically down-regulated by treatment with siRNA. mRNA levels after treatment were on average down to 16% (±5.5% SD) 48 hours after transfection. Upon incubation with 1.25 μmol/L of HU210 for 48 hours, viability of scrambled-transfected cells was reduced to 37% (±9.4 SE), whereas cells with lower p8 transcript levels showed a rescue in viability up to 63% (±8.5 SE; Fig. 5B). This suggests that an increase in p8 levels after cannabinoid treatment is an important component of the molecular response. These experiments indicate that p8 mediates, at least in part, the reduction in viability observed after cannabinoid treatment.

HU210 Reduces Tumor Growth of tposRMS Xenografts

To test whether HU210 might have a therapeutic effect on tposRMS tumors in vivo, tumor xenografts were generated by s.c. injection of Rh4 cells into immunodeficient NOG mice. Tumors were treated peritumorally with HU210 daily for 13 subsequent days. We observed significantly reduced tumor growth in HU210-treated compared with vehicle-treated animals (Fig. 6). Tumors were excised after the last day of treatment and paraffin-embedded sections were

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**Figure 3.** Cannabinoids induce apoptosis in tposRMS cells. **A**, Rh4 cells were treated for 6, 16, 24, and 48 h with either 1.25 μmol/L HU210 or DMSO. Rh28 and Rh4 cell were incubated with increasing concentrations of HU210 for 24 h (bottom). Subsequently, Western blotting was done with an anti-PARP antibody. **B**, after preincubation of Rh4 cells with 0.5 μmol/L of CB1 antagonist AM251, HU210 was added at concentrations of 1 and 1.25 μmol/L HU210 for 20 h. Cell lysates were probed with anti-PARP (top) and anti-actin (bottom) by immunoblotting. **C**, densitometric quantification of the ratio of cleaved to uncleaved PARP product (values ± SE, n = 2). **D**, percentage of cells staining positively for proapoptotic caspase-3 is shown as evaluated after 20 h of HU210 treatment of Rh4 cells (values ± SE, n = 3, significance P < 0.005). Rh4 cells were either treated with 2 and 4 μmol/L of THC (E) or 5 and 10 μmol/L of Met-F-AEA (F) for 24 h. Protein extract was analyzed for PARP and actin by Western blotting (here shown a representative blot).
either H&E stained or subsequently immunohistochemically analyzed with antibodies against the proliferation marker Ki67 and the apoptosis indicator cleaved caspase-3. H&E-stained sections from HU210-treated animals displayed a high number of cell-free patches filled with connective tissue, which are probably remains of previously apoptotic or necrotic areas. In agreement with this, a moderate increase of apoptotic cells, which was variable across tumors, was detected in HU210-treated mice compared with vehicle-treated animals (data not shown). On the other hand, no difference in the staining pattern for Ki67 was observed among treatment modalities (data not shown). In conclusion, HU210 is capable of reducing aRMS xenograft growth through induction of apoptosis in vivo.

Discussion

Evidence from in vitro and in vivo experiments suggests that cannabinoid receptor agonists can reduce tumor growth and induce apoptosis in several tumor types, including melanoma, breast and prostate cancer, colon cancer, leukemia, and glioma. However, to our knowledge, the response to cannabinoid treatment has not been studied in sarcomas yet. Here, we investigated the effects of cannabinoid receptor agonists in the sarcoma tposRMS, which we not only confirmed to express high levels of CB1 mRNA but also showed expression on the protein level by Western blot and immunohistochemistry.

In vitro, cannabinoid receptor agonists HU210, THC, and Met-F-AEA exerted an antiproliferative and proapoptotic action on tposRMS cells through activation of the CB1 receptor. The specificity of this effect for CB1 was shown by two means: First, the cell viability in fibroblasts or tnegRMS control cell lines, which express only low levels of CB1, is not affected. Second, the CB1-specific antagonist AM251 was able to significantly reduce apoptosis and partially restore cell viability. TposRMS cells were most sensitive to submicromolar concentrations of HU210, THC, and Met-F-AEA, and comparable with those observed in other cancer cells such as pancreatic cancer (20), breast cancer (22), or colon cancer (27) cells.

Key events contributing to cannabinoid-triggered induction of apoptosis in tposRMS cells are diminished AKT signaling and up-regulation of the transcription factor p8. Whereas cancer cells such as melanoma (11), colon cancer (17), and glioma (15) also experience dephosphorylation of AKT after cannabinoid stimulation, nontransformed CB1-expressing cells such as neurons react with increased phosphorylation of AKT under the same circumstances (16, 28). The key event responsible for this fundamental difference is still unknown; however, de novo ceramide synthesis (29) seems to be important for induction of apoptosis in cancer.

Figure 4. Cannabinoid receptor agonists affect AKT and ERK signaling in tposRMS cells. A, RH4 cells were incubated with 1.25 μmol/L HU210 for 30 min, 1 h, and 2 h, and cell lysates were prepared. Then, Western blotting was done with antibodies against phospho-AKT (Ser473; top left) and against phospho-ERK (bottom left). Anti-phospho-GSK (bottom right) and anti-phospho-AKT (Thr308, top right) were probed on extracts of cells treated for 2 h with 1.25 μmol/L of HU210. B, phosphorylation status of AKT at Ser473 was analyzed by Western blotting after treatment of RH4 cells with 2 and 4 μmol/L THC (top) or 5 and 10 μmol/L Met-F-AEA (bottom) for 24 h.
In comparison with other drug classes such as the broad-spectrum kinase inhibitor PKC412 investigated in our laboratory (34), HU210 treatment as single agent seems less efficient in tumor growth reduction. Nevertheless, potential use of cannabinoids as therapeutic intervention for tposRMS should still be pursued, possibly in combination with conventional chemotherapies, kinase inhibitors, or other targeted agents. Several reports indicate synergistic activity of cannabinoid receptor agonists in combination with well-established antineoplastic substances. THC was reported to act synergistically with suboptimal doses of doxorubicin or cisplatin (19), and synergism between HU210 and 5-fluorouracil was recently reported as well (27).

In summary, our results support and extend the previously shown antitumor activities of cannabinoid receptor agonists by showing proapoptotic effects of HU210, THC, and Met-F-AEA on tposRMS cells in vitro and, for
the first time, show that HU210 has tumor growth inhibiting properties in vivo. This could represent one possible novel treatment strategy that might optimize outcome in this pediatric tumor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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References

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