Published in final edited form as:

J Mol Med (Berl). 2013 April ; 91(4): 449–458. doi:10.1007/s00109-012-0957-1.

O-1602, an atypical cannabinoid, inhibits tumor growth in colitis-associated colon cancer through multiple mechanisms

Julia Kargl¹, Johannes Haybaeck², Angela Stančić¹, Liisa Andersen¹, Gunther Marsche¹, Akos Heinemann¹, and Rudolf Schicho¹

¹Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Austria

²Institute of Pathology, Medical University of Graz, Austria

Abstract

Background—Cannabinoids have antiinflammatory and antitumorigenic properties. Some cannabinoids, such as O-1602, have no or only little affinity to classical cannabinoid receptors but exert cannabinoid-like antiinflammatory effects during experimental colitis. Here, we investigated whether O-1602 shows antitumorigenic effects in colon cancer cells and whether it could reduce tumorigenesis in the colon *in vivo*.

Methods—The colon cancer cell lines HT-29 and SW480 were used to study the effect of O-1602 on viability and apoptosis. The effect of O-1602 on tumor growth *in vivo* was studied in a colitis-associated colon cancer mouse model.

Results—O-1602 decreased viability and induced apoptosis in colon cancer cells in a concentration-dependent manner (0.1–10 μ M). In the mouse model, treatment with O-1602 (3 mg/kg, i.p., 12x) reduced tumor area by 50% and tumor incidence by 30%. Histological scoring revealed a significant decrease in tumor load. In tumor tissue, O-1602 decreased levels of proliferating cell nuclear antigen (PCNA), activation of oncogenic transcription factors STAT3 and NF κ B p65 and expression of TNF- α while levels for proapoptotic markers, such as p53 and BAX increased. The *in vivo* effects of O-1602 on PCNA, BAX and p53 were also observed in colon cancer cells.

Conclusion—The data provide a novel insight into antitumorigenic mechanisms of atypical cannabinoids. O-1602 exerts antitumorigenic effects by targeting colon cancer cells as well as proinflammatory pathways known to promote colitis-associated tumorigenesis. Due to its lack of central sedation, O-1602 could be an interesting compound for the treatment of colon and possibly other cancers.

Keywords

atypical cannabinoid; colitis-associated colon cancer; apoptosis; protumorigenic

Introduction

In search for new anticancer therapies, many studies have started to focus on cannabinoids as potential antitumorigenic agents. It is now known that natural and synthetic cannabinoids

---

Corresponding author Rudolf Schicho, PhD Medical University of Graz Institute of Experimental and Clinical Pharmacology Universitätsplatz 4 8010 Graz Austria Phone: 0043 316 3807851 Fax: 0043 316 3809645 rudolf.schicho@medunigraz.at.

Disclosure The authors declare no conflict of interest

The final publication is available at www.springerlink.com

cause a cannabinoid 1 and/or 2 (CB₁ and CB₂) receptor-dependent decrease in proliferation of e.g. breast [1, 2], and intestinal cancer cells [3]. Despite the promising findings, the psychotropic activity of cannabinoids, mediated via central CB₁ receptors, may pose an obstacle to cannabinoid-based cancer treatment. These side effects could be overcome by using pharmacologically active cannabinoids that lack activity at CB₁ receptors. Indeed, certain cannabinoids, such as O-1602 and cannabidiol, have no or only little affinity to CB₁/CB₂ receptors [4, 5] and lack central sedation [6, 7], but similar to CB₁ agonists, they are able to produce typical cannabimimetic effects, such as reduction of inflammation [7] or relaxation of vascular smooth muscle [4]. We previously showed that O-1602 could reduce severity of experimental colitis independently of CB₁, CB₂ and of the atypical cannabinoid receptor GPR55 through inhibition of neutrophil migration [7]. Similar to other cannabinoids, O-1602 can also induce food intake [8] and slow down contractions of intestinal smooth muscle strips [9], suggesting that it may cause cannabinoid-like effects in the gastrointestinal (GI) tract.

Next to GI inflammation, natural cannabinoids and synthetic cannabinoid receptor ligands have been studied in carcinoma cell lines of the GI tract. In colon carcinoma, antitumorigenic activity of cannabinoids involves activation of CB₁ and CB₂ [3, 10, 11], although antiproliferative effects in colon cancer and cholangiocarcinoma cells have also been demonstrated for non-CB₁/CB₂-targeting cannabinoids, such as cannabidiol [12] and O-1602 [13]. In a recent study, cannabidiol has shown preventive effects on tumor growth in an experimental *in vivo* form of colon cancer [14].

An important hallmark in the tumorigenesis of colon cancer is inflammation [15] emphasizing that people suffering from inflammatory bowel disease (IBD) have an increased risk of developing colon cancer [16]. Colorectal tumors are infiltrated with multiple types of immune cells that can act as a source of protumorigenic cytokines like tumor necrosis factor alpha (TNF- α) and interleukin 1-beta (IL-1 β) [17]; these cytokines may contribute to mutations in tumor suppressor genes, such as p53 [17]. Thus, mutations in the p53 gene represent an early marker of neoplastic progression in ulcerative colitis [18]. Of the inflammatory cytokines, the tumor-promoting role of TNF- α is well established in colon and other cancers [19]. Additionally, many colon tumors constitutively activate transcription factors involved in inflammatory pathways, such as the signal transducer and activator of transcription factor 3 (STAT 3) and nuclear factor NF-kappa-B (NF κ B) [17]. Activation of STAT3 has been shown to play an essential role in the survival of intestinal epithelial cells and the development of colitis-associated colorectal tumors [20].

Based on our previous work in which we showed that O-1602 decreases inflammation in experimental colitis [7], we sought to investigate the effects of this compound in colon carcinoma cell lines and in a model of colitis-driven colon cancer to test whether or not it could reduce tumor growth in the colon. In the present study, we used a mouse model in which rapid development of tumors in the colon is triggered by application of the mutagen azoxymethane (AOM) and the inflammatory agent dextran sulfate sodium (DSS) [21, 22]. Antitumorigenic effects – i.e., inhibition of proliferation and induction of apoptosis – were investigated in the colon cancer cell lines HT-29 and SW480 [11].

Materials and Methods

Cell culture and drugs

The colon cancer cell lines HT-29 and SW480 (Interlab Cell Line Collection, Genoa, Italy) were cultured in McCoy's 5A media (PAA Laboratories, Pasching, Austria) and in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Grand Island, NY, USA), both supplemented with 10% FBS and 1% penicillin/streptomycin (PAA)

at 37°C in 5% CO₂ humidified atmosphere. All cell culture experiments were performed using starvation media (Opti-MEM, Invitrogen). DSS (reagent grade; 36-50 kDa) was purchased from MP Biomedicals (Eschwege, Germany) and AOM from Sigma (St. Louis, MO, USA). O-1602 (5-Methyl-4-[(1*R*,6*R*)-3-methyl-6-(1-cyclohexen-1-yl)]-1,3-benzenediol) was obtained from Tocris Bioscience (Bristol, UK) and diluted in EtOH, Tween20 and sterile saline (1:1:8).

Cell viability assay

HT-29 and SW480 cells were seeded into 96-well plates (5000 cells/well) and allowed to adhere overnight. Adherent cells were treated with increasing concentrations of O-1602 or vehicle (EtOH) in serum-free media. Compound was freshly added every 24 hours. By using the Cell Titer 96® AQueous One Solution Assay (Promega, Madison, WI, USA), cell viability was measured following the users instructions at 490nm on a BioRad xMark™ microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

Apoptosis flow cytometry and microscopy

For flow cytometric analysis of apoptosis, cells were seeded in 24-well plates and apoptosis was analyzed by using FITC-conjugated annexin V and propidium iodide (PI) staining (Invitrogen) on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). To visualize apoptosis, fluorescence microscopy was used. Briefly, cells were seeded on poly-L-lysine-coated (Sigma) coverslips, treated with O-1602 or vehicle for 72 hours. Coverslips were washed, incubated with annexin V/PI solution for 15 minutes in the dark, and additional buffer was added. Subsequently, coverslips were mounted in buffer and analyzed on a fluorescence microscope (Olympus IX 70). Images were taken using a Hamamatsu ORCA CCD camera and xcellence® Olympus imaging software (Olympus, Vienna, Austria).

Western blots

Protein expression of phosphorylated and total STAT3 (pSTAT3 and tSTAT3) and NFκB p65 (pNFκB p65 and tNFκB p65) and of proliferating cell nuclear antigen (PCNA), p53, BAX, and β-actin were detected by Western blot techniques. Protein concentration of colon tissue samples was determined by use of a Bradford assay. Lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS/PAGE) (Invitrogen) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked in TBST buffer containing 5% milk. Membranes were incubated with monoclonal mouse anti-PCNA antibody (Clone PC10; DakoCytomation, Glostrup, Denmark) and the rabbit antibodies anti-phospho-NFκB p65 (pNFκB p65; Ser536), anti-NFκB p65, anti-phospho-STAT3 (pSTAT3; Ser727), anti-STAT3, anti-p53, anti-BAX (Cell Signalling, New England Biolabs, Ipswich, MA, USA), anti-p53 CM5 (Novacastra, Leica Microsystems, Vienna, Austria), and mouse anti-β-actin (Sigma) overnight at 4°C and immunoblotted with HRP-conjugated antibodies (1:4000; Jackson ImmunoResearch, West Grove, PA, USA). ECL Western Blotting Substrate (Thermo Fisher Scientific) was used to visualize protein bands. Optical band densities were quantified with ImageJ software (NIH, Bethesda, MD, USA). pSTAT3 and pNFκB p65 band densities were normalized to tSTAT 3 and tNFκB p65 while PCNA, p53, and BAX band densities were normalized to β-actin. Values represent group means of normalized band densities.

Reverse transcriptase PCR and quantitative PCR

Tumor tissue samples were frozen in liquid nitrogen and mechanically dissociated in RNA buffer. Total RNA was then extracted using QIAshredder and RNeasy Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Reverse transcriptase PCR

(RT-PCR) was performed with 1 µg of total RNA and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Grand Island, NY, USA) for cDNA transcription. The RT-PCR program was set at 25°C for 10 min, followed by 120 min at 37°C with a terminal step at 85°C for 5 min. Quantitative PCR was performed using Fast SYBR® Green PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. The following primers were used: TNF-α (forward: 5'-CCCTCACACTCAGATCATCTTCT-3'; reverse: 5'-GCTACGACGTGGGCTACAG-3' [23]; IL-1β (forward: 5'-GGACATAATTGACTTCACCATGGAA 3'; reverse: 5'-CAGTCCAGCCCATACT TTAGGAA-3') [24]. Cyclophilin was used as a reference gene (forward: 5'-TTCCAGGATTCATGTGCCAG-3'; reverse: 5'-CCATCCAGCCATTCAGTCTT-3'). Real-time PCR data were analyzed based on the $2^{-\Delta\Delta ct}$ method [25]. The specificity of the PCR products was assessed by melting curve analyses which showed only single amplified products and agarose gel electrophoresis which revealed fragments at the expected base pair sizes.

Colitis-induced colon cancer model and O-1602 treatment

Male CD1 mice (3-5 weeks old; 20-22g; Charles River [Sulzfeld, Germany]) received a single intraperitoneal (i.p.) injection of AOM (10 mg/kg). After one week they were exposed to 2% DSS (wt/vol) for seven days. Mice were left for another week before treatment with O-1602 (consisting of 12 × 3 mg/kg i.p. injections of O-1602 or vehicle every second day over 4 weeks). In a previous study, a dose of 3 mg/kg O-1602 was determined as sufficient to induce antiinflammatory effects during experimental colitis [7]. This dose was therefore used for the treatment of mice in the present experiments. Following another period of 10 weeks, mice were sacrificed, and the colon was examined macroscopically under a dissecting microscope. The number of tumors was counted, and the tumor size (area) was measured from single tumors using a sliding caliper. For each colon, the sum of all single tumor areas was calculated. Tumor area therefore indicates the total area of all tumors measured in one colon. The colons were then either used for histopathological evaluation by forming "Swiss rolls" and fixing them in 10% phosphate-buffered formalin or for biochemical evaluation by excising tumors and adjacent tissue from the colonic mucosa. Experimental procedures were approved by the Austrian Federal Ministry of Science and Research (protocol number, BMWF-66.010/0020-II/3b/2011) and performed in accordance with international guidelines.

Histopathological analysis of tumors

After fixation in 10% phosphate-buffered formalin, colonic tissue was dehydrated, embedded in paraffin, cut on a microtome, and standard hematoxylin/eosin staining was performed on 5-µm-thick sections. Images were taken on an Olympus BX41 microscope equipped with a DP50 digital camera and xcellence® imaging software. Neoplastic characteristics were scored based on the criteria listed in Table 1.

Results

O-1602 decreases viability and promotes apoptosis of HT-29 and SW480 cells

To investigate a possible antitumorigenic effect of O-1602, we first treated the colon cancer cell lines HT-29 and SW480 with increasing concentrations of O-1602 (0.1 - 10 µM) and measured cell viability after 24 and 48 hrs. There was a significant and concentration-dependent decrease in cell viability after 24 hrs in both cell lines after incubation with 1, 5 and 10 µM of O-1602 (Figs. 1A and 1C) while a significant decrease at 0.1 µM was only seen at 48 hrs incubation time (Figs. 1B and 1D). Incubation with 5 µM O-1602 for 24 hrs decreased levels of the proliferation marker PCNA (Fig. 1E). We then tested O-1602 for its proapoptotic abilities and treated HT-29 and SW480 cells with the same concentrations of

O-1602. Also in the apoptosis assay, O-1602 displayed antineoplastic activities. As determined by flow cytometry, apoptosis was significantly increased after incubating cells with 5 or 10 μ M of O-1602 for 72 hrs (Figs. 2A and 2C). Analysis by fluorescence microscopy revealed intense and colocalized staining for annexin V and PI in both colon cancer cell lines after incubation with O-1602, indicating late apoptosis (Figs. 2 B and 2D). Incubation with 5 μ M O-1602 for 24 hours caused an increase of the tumor suppressor p53 and of the proapoptotic protein BAX (Fig. 2 E).

O-1602 inhibits tumor growth *in vivo*

Next, the effect of O-1602 on tumor growth was studied *in vivo* in the AOM and DSS-driven colon cancer model. In preliminary experiments, we investigated whether AOM or DSS alone could have induced tumor growth in the colon (n=4). No tumors developed within the entire length of the experimental time (12 weeks; data not shown). The treatment protocol and a representative image of tumors in the distal colon are shown in Fig. 3. In AOM + DSS-treated mice, macroscopic evaluation of the colon mucosa showed that O-1602 led to a more than 30% decrease in the number of tumors and an over 50% decrease in tumor area representing tumor volume reduction (Fig. 4A and 4B). The reduction of tumors was observed predominantly in the distal part of the colon where the majority of tumors were formed (Fig. 4C). We also scored tumors histologically according to the characteristics listed in Table 1 with a maximum score index of 11 (i.e., highest severity of neoplasm). The score index of AOM + DSS mice was significantly lower in the O-1602-group than in the vehicle-treated group (Fig. 5)

O-1602 has anti-proliferative and pro-apoptotic effects *in vivo*

The antineoplastic effects of O-1602 led us to investigate whether the O-1602-reduced tumor growth involved alterations in the expression of proliferation and apoptotic markers *in vivo* and whether inflammation-related mediators (i.e., protumorigenic cytokines and transcription factors) contributed to the tumor growth. Tumor cell proliferation was markedly decreased in O-1602-treated mice as determined by PCNA Western blots, while protein levels of BAX and tumor suppressor gene p53 were significantly increased (Fig. 6 A and B). Apoptosis-induced DNA fragmentation was observed in O-1602-treated mice *in situ* by TUNEL assay technique (see supplementary data, Fig. S1).

O-1602 reduces phosphorylation of NF κ B p65 and STAT3, and the expression of TNF- α in tumor tissue

Phosphorylation of NF κ B p65 and STAT3 was reduced between 40-50% in tumor tissue of AOM + DSS + O-1602 mice as compared to the vehicle-treated mice (Figs. 6A and B). The expression of protumorigenic cytokine TNF- α was reduced by around 45% whereas expression of IL-1 β did not differ between vehicle- and O-1602-treated AOM + DSS mice (Fig. 7). No significant changes were measured for these proteins in tumor-adjacent colon tissue from O-1602-treated AOM + DSS mice as compared to vehicle-treated AOM + DSS mice (see supplementary data; Fig. S2).

Discussion

Cannabis has been known for a long time to relieve symptoms of cachexia and pain associated with cancer therapy, but what has emerged is that cannabinoids themselves (natural, synthetic, and endocannabinoids) have the ability to inhibit cancer development and progression. Many of the antineoplastic effects of cannabinoids involve the activity of CB₁ and CB₂ receptors; however, non-psychoactive cannabinoids that lack affinity for CB receptors, such as cannabidiol and O-1602, are also able to inhibit proliferation of cancer cells and growth of tumors. This has been shown for cannabidiol in breast and colon cancer

[1, 2, 12, 14] and for O-1602 in cholangiocarcinoma [13], indicating that antitumor treatment using cannabinoid compounds with low affinity to CB receptors but cannabinoid-like activity is an intriguing possibility. O-1602, an atypical cannabinoid, falls into this category of compounds as it lacks CB₁ affinity [4] but exhibits antiinflammatory [7] and orexigenic properties [8] typical of CB₁ agonists.

In the current study, we first demonstrated that O-1602 reduced viability and promoted apoptosis in the cancer cell lines HT-29 and SW480. These effects were observed together with a reduction in PCNA levels and an increase in proapoptotic proteins BAX and p53. The inhibition of cell viability by O-1602 was concentration-dependent and, unlike in cholangiocarcinoma cell lines (13), it was already seen at a concentration of 0.1 μ M. In contrast, cannabidiol inhibits proliferation in CaCo-2 cells at 6-10 μ M [1]. This indicates that colon carcinoma cells may be highly sensitive to O-1602 treatment, although apoptosis was induced only after using higher concentrations (5 μ M).

To study antineoplastic effects of O-1602 *in vivo*, an AOM cancer model was employed that displays many similarities with human colorectal cancer, and that can be combined with a bout of 2% DSS-induced colitis [21]. Thus, we were able to show that O-1602, which was given at a time when early neoplasms are already present in the colon [22], significantly reduced tumor growth. Importantly, both the area and the number of tumors were markedly reduced, indicating that O-1602 interfered with tumor progression as well as with tumor initiation. The use of 2% DSS has been reported to lead to a colitis with little occurrence of flares and mucosal ulcers but with histologic inflammation of the colon [22] corroborating the concept that rather microscopic but not gross colitis determines the risk of colorectal cancer [26]. The majority of tumors in our study were observed in the distal part of the colon most likely because inflammation in the AOM + DSS model tends to be higher in this region [21]. In this respect, the model resembles the human condition of spontaneous colorectal cancer in which tumors also predominantly occur in the distal colon [15]. It is of interest that O-1602 treatment reduced tumor mass mainly in the distal colon (Fig. 4C).

The receptor involved in the antitumorigenic action of O-1602 remains uncertain. Although some *in vitro* studies have indicated that O-1602 may signal through the novel atypical cannabinoid receptor GPR55 [13, 27], this compound was clearly shown to improve experimental colitis via non-GPR55 pathways [7]. Other *in vivo* studies have also demonstrated off-target effects of O-1602 [8, 28]. As no specific GPR55 antagonist is available, an involvement of this receptor in the antitumorigenic *in vivo* effects of O-1602 remains highly speculative. It should be kept in mind that *in vitro*, O-1602 has shown agonist activity at GPR55 [27] and so far, the majority of studies suggest that an activation of GPR55 leads to an increase in proneoplastic effects (reviewed in [29]). All the results from our present study point to an antineoplastic activity of O-1602 arguing against an involvement of GPR55.

We could further show that *in vivo* treatment with O-1602 significantly decreased expression of PCNA in tumor tissue and increased BAX, corroborating our results from the *in vitro* experiments. In addition, expression of TNF- α was reduced in tumor tissue. It is well accepted that inflammation promotes colon cancer [15], and as with any inflammatory focus, colon tumor tissue is infiltrated by immunocytes that are capable of producing protumorigenic cytokines, such as TNF- α and IL-1 β [17]. IL-1 β induces expression of COX-2 in colon cancer cell lines [30], while TNF- α is known to promote spontaneous and colitis-associated colon cancer at any stage of the tumor development [19]. In a study, in which AOM + DSS mice were treated with the specific TNF- α antagonist etanercept, the number and size of colorectal tumors were widely reduced suggesting that TNF- α is involved in initiation and progression of cancer formation [31]. This raises the possibility

that downregulation of TNF- α by O-1602 could have contributed to the drug's antitumorigenicity. We did not see a significant reduction in IL-1 β which could have been caused by a differential effect of O-1602 on cytokine-producing malignant cells and cells of the tumor microenvironment, such as infiltrated leukocytes, known to release cytokines. However, not much is known yet about O-1602's actions on immune cells and cytokine expression except for that neutrophils are inhibited in their migration by the drug [7].

Other prominent effects of the O-1602 treatment *in vivo* were the reduction of phosphorylated STAT3 and NF κ B p65. STAT3 has been long regarded as an oncogenic transcription factor [32], although its tumor-promoting role in colon cancer has only recently been proven [20]. The authors demonstrated that specific knockout of STAT3 in intestinal epithelial cells interfered with tumorigenesis in a colitis-induced cancer mouse model identifying STAT3 as a crucial promoter of tumorigenesis [20]. High levels of pSTAT3, for instance, were seen in human IBD and mice with DSS colitis [33]. The role of NF κ B is well recognized in colon cancer, and it has been suggested that NF κ B is involved in early stages of tumor promotion [17]. The increased expression of p53 in tumor tissue of O-1602-treated AOM + DSS mice and in HT-29 cells incubated with O-1602 is a crucial finding. The allelic loss of p53 in colitis-associated colon cancer can be as high as 86% [34] and is an important factor in the development of colorectal cancer. In a DSS-induced colon cancer model, p53 knockout mice displayed an increase in colonic neoplasms, indicating that p53, like in humans, protects from the development of tumors [35]. The observed increase of p53 in our present study could have therefore contributed to an inhibition of cancer progression.

In conclusion, our data expand our knowledge on the role of cannabinoids in colon cancer. The antitumorigenic effects of O-1602 are multiple in that it reduces viability and proliferation of cancer cells and further promotes their apoptosis by increasing protein levels of proapoptotic BAX and the tumor suppressor gene p53. These effects are evident *in vivo* as well as *in vitro*. O-1602 also interferes with the expression of TNF- α and with the activation of STAT3 and NF κ B p65 which are key molecules that link inflammation with colon cancer. The sum of these effects most likely contributed to a reduction in tumor growth. Therefore, knowledge of O-1602's antineoplastic effects may provide an interesting basis for a new therapeutic strategy on how to treat colon and possibly other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Veronika Pommer for excellent technical assistance. This study was supported by grants from the Austrian Science Fund (P 22771 to RS, P22521 to AH, and P21004 to GM), the Austrian National Bank (OeNB 14429 to RS and 14263 to AH), the Franz Lanyar Foundation (351 to RS), and the Innovative Medicines Initiative Joint Undertaking (IMI) Grant (OncoTrack to JH). JK and AS are funded by the PhD program of the Medical University of Graz.

References

1. Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M, Di Marzo V. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J Pharmacol Exp Ther*. 2006; 318:1375–1387. [PubMed: 16728591]
2. Caffarel MM, Andradas C, Mira E, Pérez-Gómez E, Cerutti C, Moreno-Bueno G, Flores JM, García-Real I, Palacios J, Mañes S, et al. Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition. *Mol Cancer*. 2010; 9:196. [PubMed: 20649976]

- J Mol Med (Berl)*. Author manuscript; available in PMC 2014 April 01.

21. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.* 2003; 94:965–973. [PubMed: 14611673]
22. Robertis MD, Massi E, Poeta ML, Carotti S, Morini S, Cecchetelli L, Signori E, Fazio VM. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. *J Carcinog.* 2011; 10:9. [PubMed: 21483655]
23. Sakai H, Yamada Y, Shimizu M, Saito K, Moriawaki H, Hara A. Genetic ablation of TNF- α demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis. *Chem Bio. Interact.* 2010; 184:423–430. [PubMed: 20079348]
24. Lavi I, Levinson D, Peri I, Nimri L, Hadar Y, Schwartz B. Orally administered glucans from the edible mushroom *Pleurotus pulmonarius* reduce acute inflammation in dextran sulfate sodium-induced experimental colitis. *Br J Nutr.* 2010; 103:393–402. [PubMed: 19772681]
25. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
26. Mathy C, Schneider K, Chen YY, Varma M, Terdiman JP, Mahadevan U. Gross versus microscopic pancolitis and the occurrence of neoplasia in ulcerative colitis. *Inflamm Bowel Dis.* 2003; 9:351–355. [PubMed: 14671483]
27. Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol.* 2007; 152:1092–1101. [PubMed: 17876302]
28. Johns DG, Behm DJ, Walker DJ, Ao Z, Shapland EM, Daniels DA, Riddick M, Dowell S, Staton PC, Green P, et al. The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. *Br J Pharmacol.* 2007; 152:825–831. [PubMed: 17704827]
29. Ross RA. L- α -lysophosphatidylinositol meets GPR55: a deadly relationship. *Trends Pharmacol Sci.* 2011; 32:265–269. [PubMed: 21367464]
30. Liu W, Reinmuth N, Stoeltzing O, Parikh AA, Tellez C, Williams S, Jung YD, Fan F, Takeda A, Akagi M, et al. Cyclooxygenase-2 is up-regulated by interleukin-1 beta in human colorectal cancer cells via multiple signaling pathways. *Cancer Res.* 2003; 63:3632–3636. [PubMed: 12839952]
31. Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N. Blocking TNF- α in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest.* 2008; 118:560–570. [PubMed: 18219394]
32. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. *Cell.* 1999; 98:295–303. [PubMed: 10458605]
33. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, et al. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med.* 2001; 193:471–481. [PubMed: 11181699]
34. Burner GC, Rabinovitch PS, Haggitt RC, Crispin DA, Brentnall TA, Kolli VR, Stevens AC, Rubin CE. Neoplastic progression in ulcerative colitis: histology, DNA content, and loss of a p53 allele. *Gastroenterology.* 1992; 103:1602–1610. [PubMed: 1358743]
35. Chang WC, Coudry RA, Clapper ML, Zhang X, Williams KL, Spittle CS, Li T, Cooper HS. Loss of p53 enhances the induction of colitis-associated neoplasia by dextran sulfate sodium. *Carcinogenesis.* 2007; 28:2375–2381. [PubMed: 17557903]

Fig. 1. O-1602 reduces cell viability in colon cancer cells HT-29 and SW480

Adherent cancer cells were incubated with O-1602 (0.1, 1, 5, 10 μM) or vehicle (EtOH) for 24 or 48 h in serum-free media. O-1602 or vehicle was freshly added after 24 h and triplicates were used for each condition. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Cell Titer 96® AQueous One Solution Assay) and expressed as percent decrease of vehicle-treated cells which were set at 100%. HT-29 (**A**, **B**) and SW480 cells (**C**, **D**) showed a concentration-dependent decrease in cell viability after 24 and 48 hrs of incubation time. (**E**) Western Blots show a significant decrease of the proliferation marker PCNA in SW480 cells after 24 h incubation with 5 μM O-1602 (percent decrease compared to vehicle treatment [set at 100%]). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 3$; One-Way Anova and Tukey's post hoc test.

Fig. 2. O-1602 promotes apoptosis in the colon cancer cells HT-29 and SW480

Cells were incubated with O-1602 (0.1, 1, 5, 10 μM) or vehicle (EtOH) for 3 days. O-1602 was freshly added every 24 h and apoptosis was determined by FITC-conjugated annexin V and PI staining and measured on a FACSCalibur flow cytometer. Values are expressed as percentage of late apoptotic cells. Concentration-dependent apoptosis starting at 5 μM O-1602 was seen in HT-29 (A) as well as in SW480 cells (C). Apoptosis was visualized using fluorescence microscopy (B, D). Representative images showing annexin V (green) and PI (red) colocalization in cells of late apoptosis after incubation with 10 μM O-1602. Calibration bars: 30 μm. (E) Western Blots show a significant increase of BAX in both cancer cell lines and of p53 in HT-29 cells after 24 h incubation with 5 μM O-1602 (percentage increase compared to vehicle treatment [set at 100%]). * p<0.05; *** p<0.001; n = 3; One-Way-Anova and Tukey's post hoc test.

Fig.3. *In vivo* model of colitis-induced colon cancer to study the effect of O-1602 on tumor growth
 Mice were initially injected with 10mg/kg i.p. of the mutagen azoxymethane (AOM) and left for one week before starting exposure to 2% of the inflammatory agent dextran sulfate sodium (DSS) supplemented in the drinking water. Treatment with O-1602 (3 mg/kg; i.p.) or vehicle started one week after finishing DSS exposure and was continued over 4 weeks during which O-1602 was applied every second day (in total 12 x). Colons were removed at the end of week twelve and used for macroscopic, histological and biochemical analyses. The image shows several tumors in the distal colon of an AOM + DSS + vehicle-treated mouse.

Fig. 4. Effect of O-1602 on tumor number and tumor area in colitis-induced colon cancer
 Treatment with O-1602 led to a significant decrease both in tumor number (**A**) and tumor area (**B**); $n = 12$, Student's t -test. In each colon, tumors were measured individually, and the sum for all tumor areas present in one colon was calculated. The tumor area in graph (**B**) represents the mean of these sums. (**C**) Tumors were predominantly located in the distal colon. O-1602 significantly reduced tumors in the distal but not in the proximal colon. $n = 9-12$; One-Way-Anova. Tukey's posthoc test.

Fig. 5. Histological scoring of HE-stained colon sections reveals a decrease of tumor load (A) Representative section of a normal colonic specimen depicting intact crypt architecture (*scale bar*, 200 μm). (B) Representative tumor from an AOM + DSS + vehicle-treated mouse with tubular growth pattern and severe cellular polymorphism resembling human tubular adenoma with high-grade intraepithelial neoplasia (high-grade dysplasia depicted by *arrows*) (*scale bar*, 500 μm). (C) Almost completely cured tumors in AOM + DSS + O-1602-treated mice. The hyperplastic colonic mucosa (depicted by *arrowheads*) exhibits less branching and budding in crypts and only reactive cytological changes (*scale bar*, 500 μm). (D) The histoscore index indicates a significant decrease in tumor load in the O-1602-treated group; n = 9-12; Student's t-test.

Fig. 6. *In vivo* treatment with O-1602 decreased transcription factors pSTAT3 and pNFκB p65 and proliferation marker PCNA while raising levels of proapoptotic p53 and BAX

(A) For *in vivo* experiments, Western blots were performed from tumor tissue using antibodies against phosphorylated STAT3 (pSTAT3), phosphorylated NFκB p65 (pNFκB p65), PCNA, p53, and BAX. Two samples of each group are shown as representative examples. (B) Graphs show the decrease of pSTAT3, pNFκB p65 and PCNA and the increase of p53 and BAX in O-1602-treated animals ($n = 6-12$; One-Way-Anova; Tukey's post hoc test). Band densities of pSTAT3 and pNFκB p65 were normalized against total STAT3 (tSTAT) and total NFκB p65 (tNFκB p65), respectively. Band densities of PCNA, p53 and BAX were normalized against β-actin.

Fig. 7. Treatment with O-1602 decreased expression of TNF- α *in vivo*

Tumor tissue was also collected for evaluation of cytokine expression. mRNA expression of TNF- α was found decreased by 45% in tumor tissue while O-1602 treatment did not change expression of IL-1 β mRNA (F) (n = 6; One-Way Anova; Tukey's post hoc test).

Table 1

Histologic criteria of tumors		Score
Number of lesions	0-2	1
	3-5	2
	6-8	3
	9-	4
Crypts	normal	0
	goblet cell depletion	1
	branching	2
	complex budding	3
Epithelium	normal	0
	hyperplasia/ACF	1
	low-grade dysplasia	2
	high-grade dysplasia	3
Submucosal invasion	absent	0
	present	1

ACF, aberrant crypt foci