Cytotoxic effect of efavirenz is selective against cancer cells and associated with the cannabinoid system

Markus Hecht[a], Thomas Harrer[b], Maike Büttner[c], Manuela Schwegler[a], Sonja Erber[a], Rainer Fietkau[a] and Luitpold V. Distel[a]

Background: Recently, a regression of precancerous lesions in HIV-1-infected patients after initiation of HAART was reported. Nonnucleoside reverse transcriptase inhibitors (NNRTIs) as efavirenz (EFV) might be mediators of this effect, as they are known to have a cytotoxic effect on tumour cells. A potential mechanism involved in this effect may be the activation of the cannabinoid receptor to mediate tumour toxicity.

Methods: Several tumour-derived and fibroblast cell lines were studied. Cytotoxicity of EFV was evaluated by Annexin-Pi staining. The expression of the cannabinoid receptors CB1, CB2 and GPR55 was analysed by western blot, quantitative reverse transcriptase (qRT-PCR) and fluorescence activated cell sorting. The influence of the cannabinoid agonists and antagonists on the effects of EFV was investigated. Furthermore, the effect of EFV on the phosphorylation state of the growth factors Erk, Akt and the tumour suppressor protein p53 was tested.

Results: EFV revealed a selective cytotoxic effect on several tumour cell lines, whereas primary fibroblasts were not affected. The cytotoxic effect was associated with the expression of CB1. The combination of EFV with cannabinoid agonists showed an increase in toxicity. The phosphorylation state of Erk and Akt was not affected by EFV, whereas p53 showed an increased phosphorylation.

Conclusion: EFV has a selective cytotoxic effect on several tumour cells. Furthermore, EFV led to an activating phosphorylation of the tumour suppressor protein p53 going in line with earlier reports that EFV may be antitumourigenic and a potential cytostatic drug. The observed synergistic effect with cannabinoid agonists implicates an involvement of the cannabinoid system.

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Introduction

Since the introduction of HAART, the survival of HIV-1-infected patients is prolonged and causes of death have changed. Nowadays, about one-third of all deaths in HIV patients are cancer related, with an increase of non-AIDS-related malignancies [1]. Consequently, cancer prophylaxis will be one of the future challenges in HIV medicine. Data exist about a decreased incidence of precancerous lesions in HIV-1-infected patients on HAART compared with patients without HAART [2–4]. Recently, even regressions of precancerous cervical lesions in women after initiation of HAART in prospective cohorts were reported [5,6]. The

[a]Department of Radiation Oncology, [b]Department of Internal Medicine 3, and [c]Department of Pathology, University Hospital Erlangen, Friedrich-Alexander-University of Erlangen-Nuremberg, Erlangen, Germany.

Correspondence to Markus Hecht, Department of Radiation Oncology, University Hospital Erlangen, Friedrich-Alexander-University of Erlangen-Nuremberg, Universitätsstraße 27, D-91054 Erlangen, Germany.

Tel: +49 9131 854 4247; fax: +49 9131 853 9335; e-mail: Markus.Hecht@uk-erlangen.de

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conclusion of these results is either a cancer prophylaxis due to immune reconstitution or an antineoplastic effect of the drugs used in HAART. A cytotoxic effect of nonnucleoside reverse transcriptase inhibitors (NNRTIs) on tumour cell lines *in vitro* has recently been shown [7]. Therefore, we assumed antineoplastic effects of the NNRTI efavirenz (EFV) to been involved in the protection against tumourigenesis. In high concentrations, EFV might even be a useful chemotherapeutic drug in cancer patients. Use of EFV yields a positive drug screening test for cannabis in some HIV-1-infected patients [8–11]. The cytotoxic effect of cannabinoids on tumour cells has often been shown [12–15]. This effect might be based on several different mechanisms, including the inhibition of growth factor signalling (Akt, Erk, Jnk), Ceramide de-novo synthesis, lipid raft-dependent mechanisms resulting in endoplasmatic reticulum stress, decreased mitochondrial membrane potential and mammalian target of rapamycin complex 1 (mTORC1)-triggered endocytosis [16–19]. Taken together, we hypothesize that EFV is directly toxic on tumour cells and this effect may be caused via the signalling pathways of cannabinoids.

Materials and methods

Cell culture

Cell lines were cultured at 37°C in a 5% CO_2 incubator. The tumour cell lines T98G (glioblastoma), HCT-15 (colorectal carcinoma), BxPC-3 (pancreatic carcinoma) and Panc-1 (pancreatic carcinoma) were grown in Dulbecco's Modified Eagle Medium (PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% foetal bovine serum. The Jurkat cell line (acute T-cell leukaemia) was cultured in RPMI 1640 Medium (Biochrom AG, Berlin, Germany) supplemented with 10% foetal bovine serum. The primary human fibroblasts cell lines SBL-5 and SBL-6 were isolated from healthy individuals and grown in F-12 Medium (Gibco; Life Technologies GmbH, Darmstadt, Germany) supplemented with 12% foetal bovine serum and 2% nonessential amino acids. p25 and p26 are primary cell lines isolated from a head and neck squamous cell carcinoma [20], whereas p25 consists of fibroblasts (same medium as fibroblasts), and p26 consists of the corresponding carcinoma cells (same medium as tumour cell lines). All media were supplemented with 1% L-Glutamine and 1% penicillin/streptomycin.

Drugs

EFV, tenofovir (TDF), emtricitabine (FTC), rimonabant/SR141716 (all Sequoia Research Products Limited, Pangbourne, UK) and Win 55212-2 (Cayman Chemical Company, Ann Arbor, Michigan, USA) were dissolved in DMSO. (−)11-nor-carboxy-Δ^9-THC (Cayman Chemical) was dissolved in methanol.

Flow cytometry

Apoptosis and necrosis was detected by the Annexin-V-FITC and propidium iodide assay. Briefly, cells were suspended in Ringer solution and stained with Annexin-V-FITC (0.4 μg/ml) and propidium iodide (48 ng/ml) for 30 min at 4°C as reported before [21]. Cell cycle analysis was performed by propidium iodide Triton staining. Cells were incubated for 24 h at 4°C in a mixture of the detergent Triton X-100, Na-citrate and propidium iodide (1 μg/ml). Receptor expression of cells was estimated by flow cytometry. Cells were suspended in PBS supplemented with EDTA. Primary antibodies against CB1 (Abcam plc, Cambridge, UK), CB2 (Abcam) and GPR55 (Abcam) were added for 30 min at room temperature. After washing, Alexa labelled secondary antibodies (Invitrogen; Life Technologies GmbH, Darmstadt, Germany) were added. Experiments were performed twice with three replicates per run. A Gallios flow cytometer (Gallios Cytometer 1.1 Software; Beckmann Coulter GmbH, Krefeld, Germany) was used. Results were analysed with Kaluza Flow Cytometry Analysis 1.1 (Beckmann Coulter).

Immunostaining

Immunostaining was performed following a standard protocol described before with two modifications [22]. Cells were transferred onto a glass slide by cytocentrifugation and were fixed without Triton X-100. The same primary and secondary antibodies as for flow cytometry were used. Images were captured by fluorescence microscopy as greyscale images (Leica DM 6000). Overlays were build using an image processing software (Biomas 3.3 10/2004 MSAB).

Western blot

Protein of whole cells was extracted by radio-immunoprecipitation assay buffer. Isolation of cytosolic and nuclear protein was performed with NE-PER Kit (Thermo scientific, Bonn, Germany). The protocols for the protein isolation and the following western blot have been described before [22,23]. The following primary antibodies were used: anti-CB1, anti-CB2, anti-GPR55, anti-beta Actin (all Abcam), anti-pERK1/2 Thr202/Tyr204 (Santa Cruz Biotechnology, Santa Cruz, California, USA; Abcam), anti-pERK1/2 Thr177 (Santa Cruz), anti-ERK2 (Santa Cruz), antipAkt Thr308 (Santa Cruz), anti-pAktSer473 (Cell Signaling Technology Inc., Danvers, Massachusetts, USA), anti-Akt1/2/3 (Santa Cruz). Horseradish peroxidase-labelled secondary antibodies were used: anti-mouse (Abcam), anti-rabbit (Abcam), anti-goat (Santa Cruz). Western blots were analysed semi-quantitatively by Biomas Software.

Quantitative reverse transcriptase PCR

Total RNA was isolated from various cell lines using Qia shredder and RN easy mini kit (Qiagen Sciences, Germantown, Maryland, USA) according to the manufacturer's instructions. cDNA was created using Quanti

Tectreverse transcription kit (Qiagen) followed by Sybr Green real-time qPCR experiments carried out with Sso Fast Eva Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany). Gene study was completed with CFX Manager Software (BioRad), normalizing to control levels of *RPL29* mRNA levels. The following primers (5′-3′) were used: *CB2* forward catgctgtgcctcatcaact; *CB2* reverse gggcttcttcttttgcctct; *GPR55* forward tctccctcccattcaagatg; *GPR55* reverse gtccatgctgatgaagcaga; *RPL29* forward TCCGGCGTTG TTGACCCTATTTC; *RPL29* reverse GCCATGTCT GCACCATAAGCC.

Immunohistochemistry

Immunohistochemistry was performed following a standard protocol [24,25]. Two tissue microarrays (TMAs) were constructed, one from a panel of normal tissues and a second from different cancer tissues. TMAs were stained with an anti-CB1 (Abcam) antibody.

Statistics

Statistics were performed with IBM SPSS Statistics 19.0 for Windows (IBM Corporation, New York, New York, USA) using the two-sided *t*-test. Significant differences with a *P* value of less than 0.05 are marked as *, and highly significant differences with a *P* value of less than 0.01 are marked as **.

Results

Toxicity of nonnucleoside reverse transcriptase inhibitor efavirenz compared with nucleoside reverse transcriptase inhibitors

As HAART, frequently consisting of a combination of NRTI and NNRTI, was shown to be associated with antitumoural effects, the influence of NRTI (TDF and FTC) and the NNRTI EFV on the survival of tumour cell lines was investigated to find out which component of the medication might mediate the antitumoural effects. Cytotoxicity of EFV, TDF and FTC (single agents and combinations) on the fibroblasts SBL-5 and the pancreatic cancer cell line BxPC-3 was determined *in vitro* by analysing apoptotic and necrotic cell death using Annexin V-FITC/Pi staining (Fig. 1). Annexin V-FITC/Pi double-negative cells were considered as viable cells, Annexin V-FITC-positive/Pi-negative cells were considered as apoptotic cells and Annexin V-FITC/Pi double-positive cells were considered as necrotic cells [26]. Fibroblasts were not affected by any drug (Fig. 1a,b). In pancreatic cancer cells, necrosis was strongly induced after EFV treatment comparable with the effect of the triple combination (EFV, TDF and FTC) (Fig. 1c,d). TDF or FTC alone or in combination did not increase the basal level of apoptosis or necrosis. Cell cycle and sub-G1-DNA content was analysed by propidium iodide Triton staining. The cell cycle of fibroblasts SBL-5 was not affected by the drugs (Fig. 1e,f). EFV induces a G1 arrest in the BxPC-3 cells (Fig. 1h,k). TDF or FTC did not block the cell cycle. Sub-G1-DNA cells are regarded as being apoptotic or necrotic due to their fragmented DNA [27]. Again, EFV had a highly significant effect on the amount of sub-G1-DNA in BxPC-3 cells, but not on SBL-5 fibroblasts (Fig. 1g,l). No such effect was seen for the NRTI alone.

Efavirenz toxicity in different tumour types and fibroblasts

The results above raised the question whether EFV has a cytotoxic effect on other tumour cell lines and whether this effect is specific for neoplastic cells and spares nonneoplastic normal tissue cell lines. Six tumour cell lines and three primary fibroblast cell lines were treated with increasing concentrations of EFV and stained with Annexin V-FITC/Pi. Even a low concentration of 40 μmol/l EFV induces apoptosis and necrosis in the tumour cell lines T98G (glioblastoma), HCT-15 (colorectal carcinoma), BxPC-3 (pancreatic carcinoma), Panc-1 (pancreatic carcinoma) and Jurkat (acute T-cell leukemia). The two fibroblast cell lines SBL-5 and SBL-6 were only slightly affected at concentrations of 80 μmol/l (Fig. 2a). Furthermore, the primary head and neck squamous cell carcinoma cell line p26 and a corresponding primary oral fibroblast cell line p25 from one patient were compared in order to rule out interindividual genetic differences [20]. In the tumour cells, a strong cytotoxic effect of EFV was detected, whereas only much higher concentrations of EFV had a toxic effect on oral fibroblasts (Fig. 2b).

Correlation between efavirenz toxicity and cannabinoid receptor expression

A crucial role of the cannabinoid system in the toxicity of EFV was assumed. The first step of signal transduction is the binding to a receptor. Thus, the expression of the two established receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), and the recently described G-protein coupled receptor 55 (GPR55) were examined by western blot [28]. CB1 protein was highly expressed in the tumour cell lines and only mildly in the fibroblasts cell lines (Fig. 3a,b). A slight expression of CB2 protein was detected only in Jurkat cells. GPR55 protein expression was not detected. Due to the low detection of CB2 and GPR55, additional quantitative reverse transcriptase PCR (qRT-PCR) experiments to detect mRNA levels were performed (Fig. 3c). This confirmed the expression of CB2 in Jurkat cells only. In contrast to the results of western blotting, GPR55 mRNA was detected in T98G cells. For further investigations, the EFV-sensitive cell line T98G (glioblastoma) and the quite resistant cell line SBL-5 (fibroblasts) were chosen. The pattern of receptor expression found in western blotting was confirmed by flow cytometry and immunostaining for both cell lines (Fig. 3d,e).

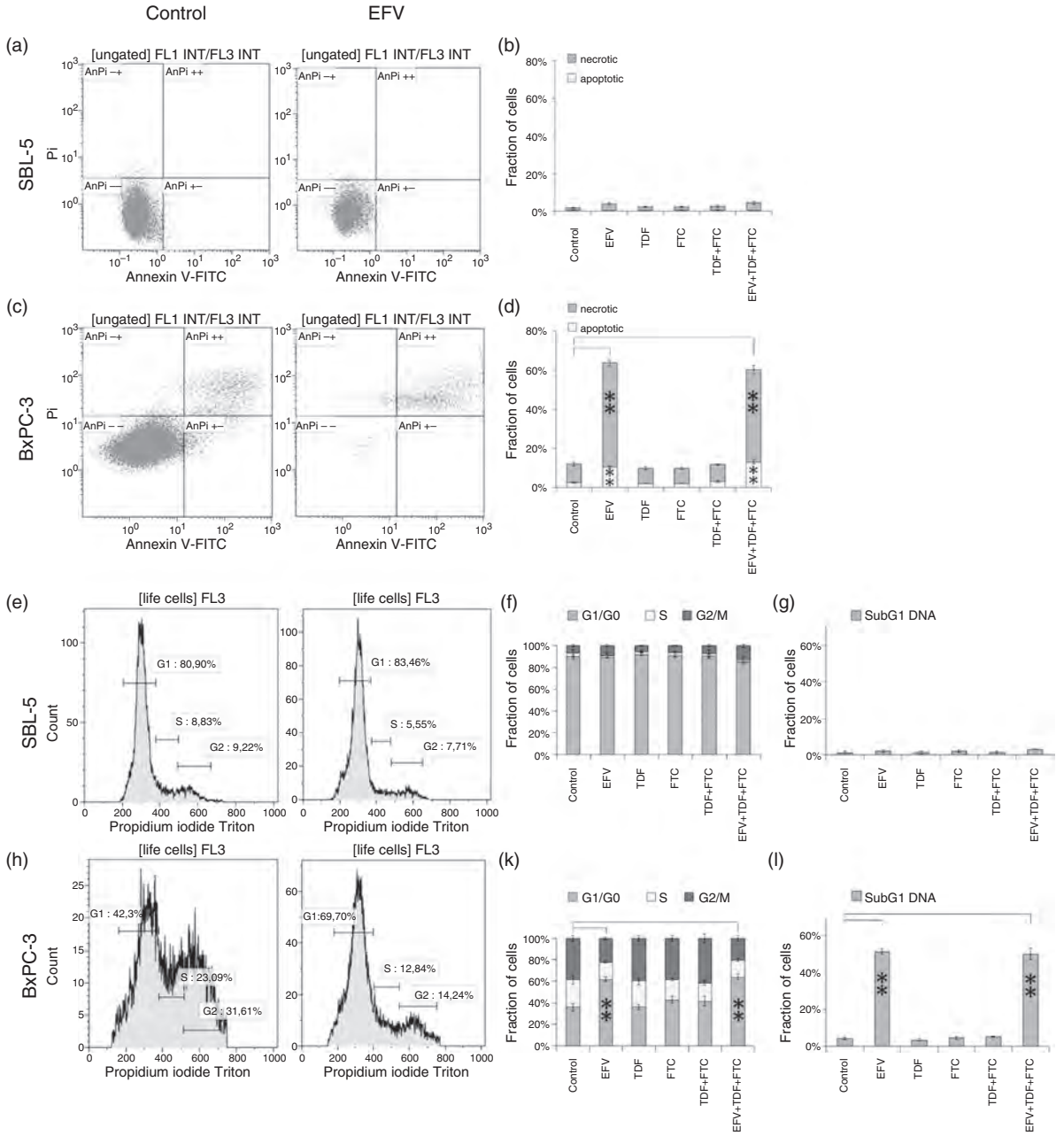

Fig. 1. Flow cytometric analysis of cytotoxicity and cell cycle effects of efavirenz, tenofovir and emtricitabine. The cells were treated for 72 h with a concentration of 60 μmol/l of each drug. Apoptosis and necrosis in (a, b) SBL-5 fibroblasts and (c, d) BxPC-3 pancreatic cancer cells was detected by Annexin V-FITC/Pi staining. Cell cycle phase of (e–g) SBL-5 fibroblasts and (h–l) BxPC-3 pancreatic cancer cells was detected by propidium iodide Triton staining. (e, f, h, k) was gated on living cells of the forward/sideward scatter diagram and (g, l) on all cells. Significant differences with $P < 0.05$ are marked as *, and highly significant differences with $P < 0.01$ are marked as **.

Expression of cannabinoid receptor 1 in tumours and normal tissue *in vivo*

To validate the results obtained in cell lines in human tissue, paraffin-embedded tissue specimens were investigated. The cannabinoid receptor expression was evaluated in 12 tumour samples and 12 normal tissue samples on two microarray slides (TMA) stained for CB1. A high CB1 expression was found in pheochromocytoma, papillary renal cell carcinoma and Barrett's carcinoma of the oesophagus (Table 1). Normal tissues were negative for CB1 with the exception of the cerebellum. The histological images of healthy renal tissue and three renal tumours are illustrated (Fig. 3f–j). The signal distribution suggests an intracellular pattern rather than a membrane pattern. This goes in line with existing data showing the localization of CB1-receptor both on the cellular membrane and on intracellular membrane systems [29,30].

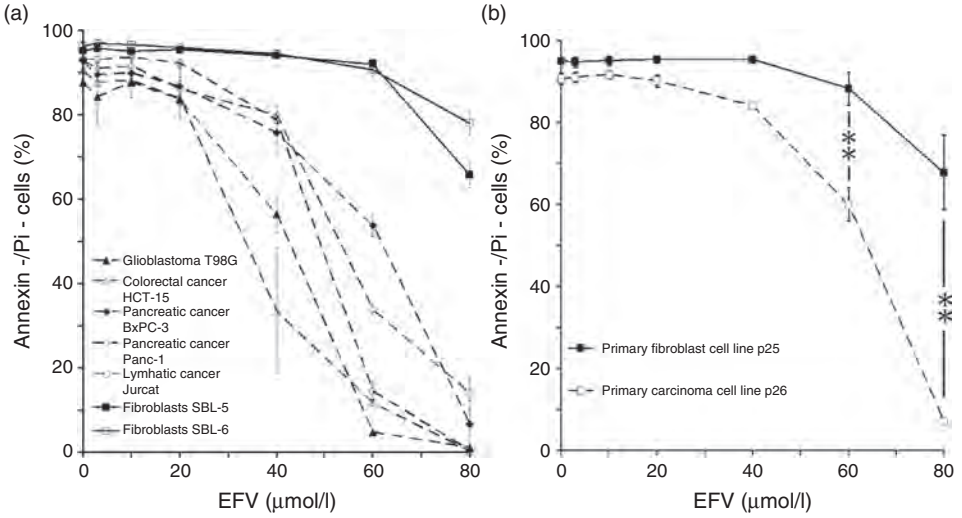

Fig. 2. Flow cytometric analysis of efavirenz cytotoxicity in different cell lines. The cells were treated with different concentrations of EFV for 72 h. (a) Annexin V-FITC/Pi double-negative cells of different fibroblasts (solid lines), tumour cells (dashed lines), (b) of primary fibroblasts (solid line) and a primary squamous carcinoma cell line (dashed line) from one individual. Significant differences with $P < 0.05$ are marked as *, and highly significant differences with $P < 0.01$ are marked as **.

Influence of cannabinoid agonists and antagonists on cytotoxicity of efavirenz

So far, only an association of EFV toxicity and cells expressing CB1 has been observed. To show a coherence of EFV toxicity and cells expressing CB1, the SBL-5 fibroblasts and the T98G glioblastoma cells were treated with combinations of EFV and a cannabinoid agonist [Win 55212-2 or (-)-11-nor-carboxy-Δ^9-THC (THC)] or an antagonist (Rimonabant/SR141716) [12]. The concentration of EFV was chosen to be mildly toxic for tumour cells and the agonist/antagonist was added in concentrations below toxicity. First, the toxicity of the individual agents was tested. THC showed no toxicity even at high concentrations. The cannabinoid agonist Win 55212-2 and the cannabinoid antagonist rimonabant were toxic above 10 μmol/l (Fig. 4a). A possible reason for the differences in the toxicity of the two cannabinoid agonists THC and Win 55212-2 might be the activation of the GPR55 receptor on T98G cells. Only THC but not Win 55212-2 is able to activate this receptor [31,32]. The combination of EFV and THC increased the toxicity significantly in T98G glioblastoma cells (Fig. 4b). Also, Win 55212-2 was distinctly toxic in combination with EFV in T98G glioblastoma cells (Fig. 4c). Blocking the CB1 receptor with rimonabant led to a mild and nonsignificant increase of the EFV-induced toxicity (Fig. 4d). Identical concentrations of the different drug combinations had no toxic effect on the fibroblasts SBL-5.

Effect of efavirenz on Erk, Akt and p53-phosphorylation

In the following, the effect of EFV on the intracellular signal transduction was further analysed. Intracellular downstream factors of cannabinoid receptors are the growth factors Erk and Akt, which are activated via phosphorylation [17–19,33,34]. The hypothesis was that EFV may lead to a dephosphorylation of the constitutively activated Erk or Akt. Therefore, T98G cells were treated with EFV for different time periods and two phosphorylation sites of Erk (Fig. 4e) and Akt (Fig. 4f) were investigated. EFV did neither influence Erk nor Akt phosphorylation at any phosphorylation site at any time. In addition, the influence of EFV on the tumour suppressor protein p53 was studied. T98G cells were treated with EFV and cellular protein was isolated separately in a cytoplasmatic and nuclear fraction. p53 was located in the nucleus and phosphorylation at Ser15 was increased with a clear EFV dose dependency (Fig. 4g).

Discussion

Selective cytotoxic effect of efavirenz on tumour cells

In-vitro data showing the cytotoxicity of NNRTI on tumour cell lines exist [7,35–38]. This promotes the idea to use EFV either as a protective drug against the development of precancerous lesions or in patients who have already developed cancer. The main characteristic of a chemotherapeutic drug must be its toxicity against tumour cells and a tolerable damage in normal tissue. The NNRTI EFV showed a clear selective antitumour cytotoxic effect in several tumour cell lines, whereas the NRTI TDF and FTC showed no cytotoxicity. The predominant type of cell death was necrosis. This may be an additional advantage, as necrotic cells trigger an inflammatory antitumour response by activating the adaptive immune system [39]. Moreover, the NNRTI EFV led to an accumulation of tumour cells in the G0/G1-phase, which was already demonstrated in other cancer cell lines [7,37]. Again, the normal tissue cell lines

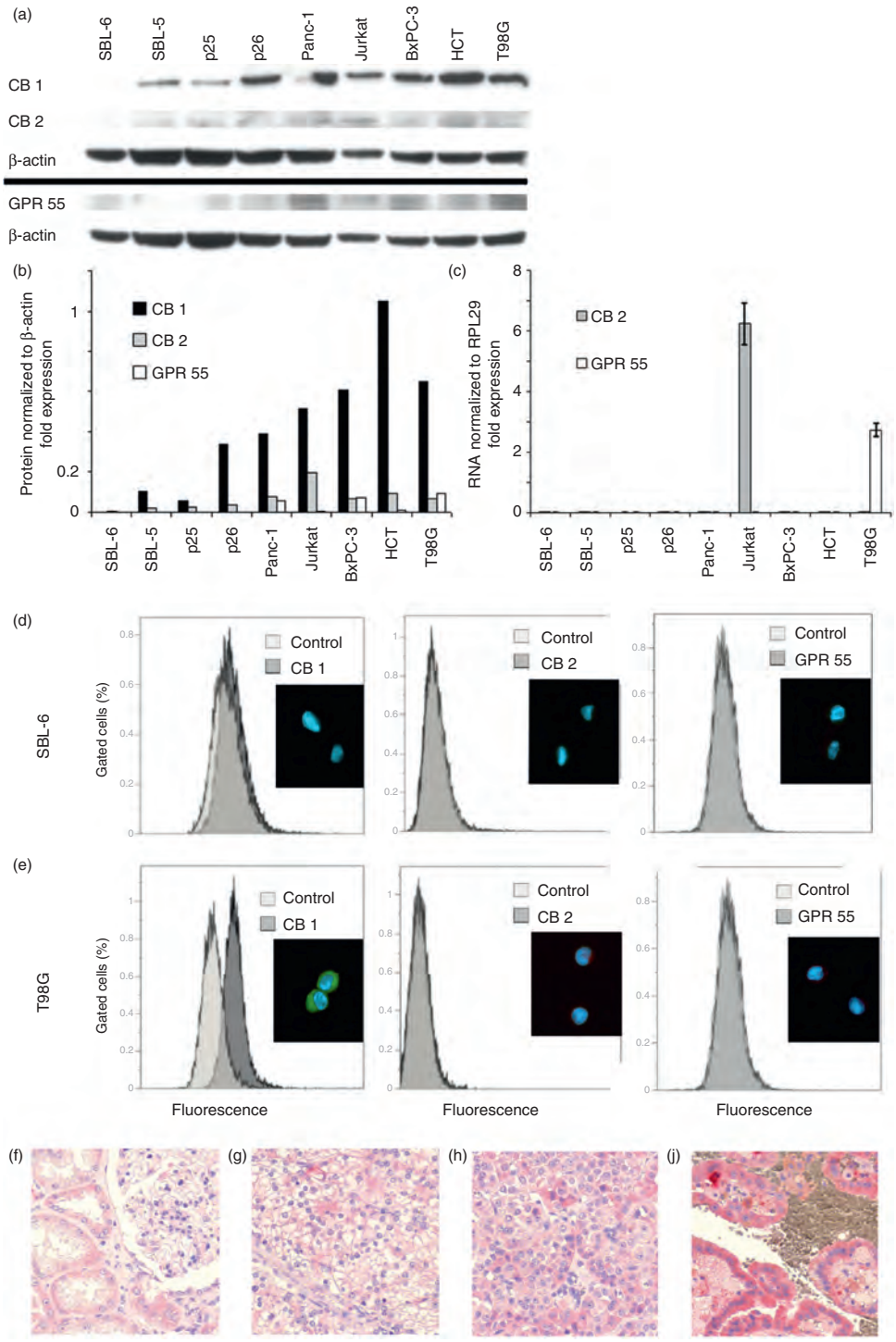

Fig. 3. Expression of cannabinoid receptors in different cell lines and tissues. (a) Whole cellular protein from nine cell lines was immunoblotted. CB1 (53 kDa) and CB2 (40 kDa) were stained on the same membrane after stripping procedure. Another membrane was stained for GPR55 (37 kDa). (b) The ratio between the amount of CB1, CB2 or GPR 55 and beta-Actin was calculated. (c) RNA was isolated from nine cell lines and a qRT-PCR was performed. mRNA levels of CB2 and GPR 55 were normalized to RPL29. (d) SBL-5 fibroblasts and (e) T98G glioblastoma cells were stained for the cannabinoid receptors CB1, CB2 and GPR55 with fluorescence-marked antibodies and analysed by flow cytometry. (f) Normal renal tissue, (g) clear cell renal carcinoma, (h) oncocytoma and (j) papillary renal cell carcinoma were stained for CB1.

Table 1. Expression of cannabinoid receptor 1 *in vivo*.

Normal tissue microarray				Tumour tissue microarray			
Colon	–	Kidney	–	Gastric carcinoma	–	Papillary renal cell carcinoma	+
Small intestine	–	Skin	–	Colon carcinoma	–	Sarcoma	–
Suprarenal gland	–	Uterus	–	Clear cell renal carcinoma	–	Ovarian carcinoma	–
Prostate	–	Testes	–	Oncocytoma	–	Melanoma	–
Ovary	–	Cerebellum	+	Pheochromocytoma	+	Barrett's carcinoma	+
Liver	–	Pancreas	–	Prostate carcinoma	–	Endometrial carcinoma	–

A normal tissue microarray and a tumour tissue microarray were stained for CB1. Tissues with an expression of CB1 are marked with (+), and tissues without CB1 are marked with (–).

were not affected. Furthermore, there exist data about a reduced growth of human tumour xenografts in nude mice under therapy with EFV [37]. The severe toxicity on tumour cells at low concentrations and the almost complete sparing of the fibroblasts gives reason to expect a wide therapeutic range of EFV *in vivo*.

Influence of the cannabinoid system on efavirenz cytotoxicity

So far, the mechanism of action causing the selective antineoplastic effect of EFV is not well understood. The group around Spadafora suggested the existence of a reverse transcriptase enzyme in tumour cells. It is concluded that NNRTIs, but not NRTIs, inhibit this enzyme, which leads to cytotoxicity [7,35,36]. The thesis of this article is based on the observation that EFV can cause a positive drug screening for cannabis in patients [8–11]. Furthermore, the side effects of EFV with dizziness, somnolence and abnormal dreams show parallels to the side effects of orally administered THC (Tetrahydrocannabinol) [40–42]. In addition, cannabinoids are toxic against tumour cells [12–15] and lead to a G1/G0 cell cycle arrest as EFV does [43]. Consequently, we speculated that the antineoplastic effect of EFV could be mediated via cannabinoid pathways.

The main cannabinoid receptor types are the cannabinoid receptor 1 (CB1), the cannabinoid receptor 2 (CB2) and the G-protein coupled receptor 55 (GPR55) [28]. The CB1-receptor was found on all investigated tumour cells lines but not on the three different fibroblasts cell lines. CB2 was only detected in Jurkat cells. GPR55 protein could not be detected in any of the cell lines, whereas GPR55 mRNA was found only in T98G cells. These results are contrary to recent findings implicating a crucial role of CB2 in glioma cells [18]. To address the question whether the CB1-receptor is expressed in tumours *in vivo*, paraffin-embedded normal tissue and tumour tissue was investigated by CB1-receptor immunohistochemistry. In normal tissues, with exception of the cerebellum, no CB1 expression was found. In contrast, some tumours showed a high CB1 expression *in vivo*. It was shown before that the CB1-receptor immunoreactivity was found to be an adverse prognostic factor in several tumour entities *in vivo* [44,45].

So far, only a correlation of EFV toxicity and the CB1 receptor expression has been demonstrated. In order to prove a mechanism of action, the rate of cell death was studied with EFV in combination with the cannabinoid agonists THC and Win 55212-2 and an antagonist Rimonabant (SR141716) [12]. In this setting, a significant increase of the EFV toxicity in combination with the cannabinoid agonists was found. However, the mild and not significant increase of the EFV toxicity by the cannabinoid antagonist argues against a direct receptor-mediated mechanism. The cytotoxicity of Rimonabant against tumour cells has been reported before, whereas mechanisms such as the inhibition of growth signalling, mitotic DNA-damage, caspase-dependent mechanisms and lipid-raft mediated mechanism were discussed [46–48]. In summary, the increased tumour toxicity of EFV by the agonists suggests synergistic mechanisms of EFV toxicity with the cannabinoid system or its downstream signalling. It seems improbable that this is mediated directly through the cannabinoid receptor.

The growth factors Erk (extracellular signal regulated kinase) and Akt (protein kinase B) are two of the downstream signalling proteins of the cannabinoid receptors and are activated by phosphorylation [17–19,33,34]. In renal cell carcinoma cell lines, a dephosphorylation of the growth factor Erk by EFV has already been shown [49]. In the glioblastoma cell line T98G, no influence of EFV on Erk or Akt phosphorylation at any phosphorylation site could be seen. The signalling might take place via other growth factors (e.g. JNK), ceramide de-novo synthesis and lipid raft dependent mechanisms, which result in endoplasmatic reticulum stress, decreased mitochondrial membrane potential or mTORC1-triggered endocytosis [16–19]. Furthermore, the effect of EFV on the phosphorylation of the tumour suppressor protein p53 was studied. An increase of phosphorylation was shown, which leads to the activation of the protein and could be the reason for the cell cycle arrest in G1-phase [50,51]. An activation of p53 by cannabinoids has been reported before, but the mechanism is not clear [16,52].

Influence of efavirenz on precancerous lesions and cancer *in vivo*

One critical point is whether the concentrations used *in vitro* can also be achieved *in vivo* in human individuals. A severe cytotoxic effect on tumour cells was observed at a

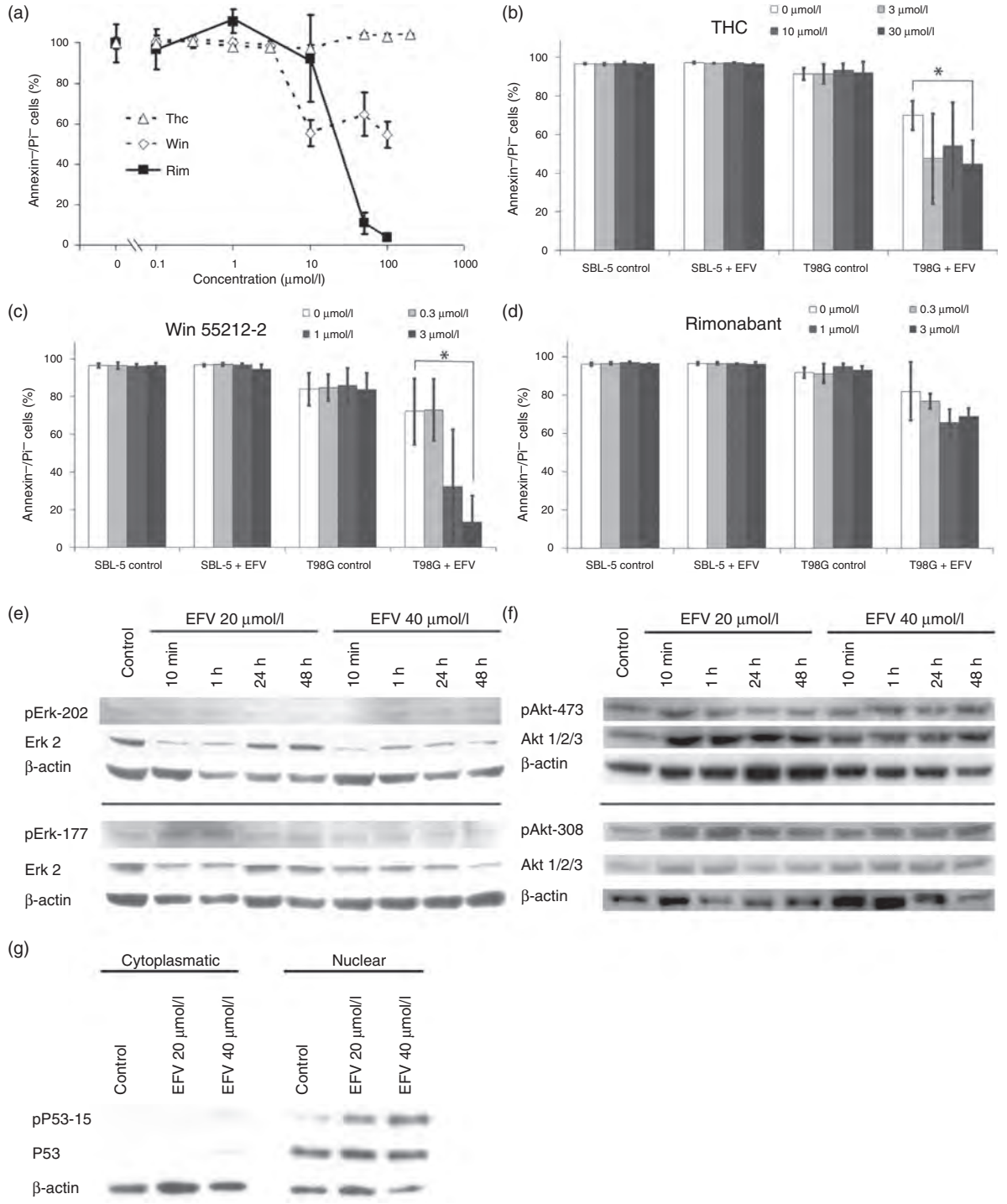

Fig. 4. Influence of cannabinoid agonists and antagonists and effects of efavirenz on intracellular signal transduction. (a) Apoptosis and necrosis induction by THC, Win 55212-2 and Rimonabant alone was detected in T98G glioblastoma cells by Annexin V-FITC/Pi staining. Different concentrations of (b) THC, (c) Win 55212-2 or (d) Rimonabant were combined with EFV 40 μmol/l for 72 h in T98G glioblastoma cells and SBL-5 fibroblasts. (e) T98G cells were treated with EFV and whole cellular protein was immunoblotted. Phosphorylation of Erk1/2 at position Thr202/Tyr204 (42/44 kDa) and Thr177 is compared with Erk2 (42 kDa) and beta-Actin (43 kDa). The different proteins were stained on the same membrane after stripping procedure. (f) Phosphorylation of Akt (55 kDa) at positions Thr308 and Ser473 is compared with Akt 1/2/3 and beta-Actin. (g) T98G glioblastoma cells were treated with EFV for 72 h and nuclear and cytosolic protein was separately immunoblotted. Phosphorylation of p53 at position Ser15 is compared with whole p53 protein and beta-Actin. Significant differences with $P < 0.05$ are marked as *, and highly significant differences with $P < 0.01$ are marked as **.

concentration of 40 μmol/l of EFV. In the therapeutic drug monitoring, the optimal range of EFV is between 1000 and 4000 μg/l (equivalent 3.2–13 μmol/l). An analysis in 130 patients under therapy with EFV revealed blood levels of more than 10 000 μg/l (equivalent 32 μmol/l) in 5% of all patients approximately 12 h after intake [53]. Studies about HIV-1-infected patients with tuberculosis worked with 800 mg EFV daily due to the combination with the cytochrome P450 inductor Rifampicine. In these studies, maximal blood concentrations of up to 20 000 μg/l (equivalent 64 μmol/l) were reported in single patients. Studies about pharmacokinetics showed a maximum concentration of EFV approximately 5 h after intake [54,55]. Consequently, the maximum concentration in patients will be even higher than reported above. *In vitro*, the tumour cells were treated for 72 h. The permanent treatment *in vivo* may also enhance the antineoplastic effect of EFV. Therefore, it appears well possible that antineoplastic levels of EFV could be reached in the patients without exceedingly increasing the EFV concentrations already used for HIV therapy.

As mentioned in the Introduction, several studies describe regression of precancerous cervical lesions in HIV-1-infected patients after initiation of HAART [5,6]. In the tumourigenesis of cervical cancer, the human papilloma virus (HPV) infection is an essential step, because HPV protein E6 leads to a degradation of the tumour suppressor protein p53 [56]. Consequently, the lack of p53-mediated cell cycle arrest, apoptosis and senescence dramatically enhances the risk of precancerous lesions and cancer development [57]. As shown above, EFV leads to an activation of p53 via phosphorylation. Furthermore, it has been demonstrated that a reactivation of p53 can lead to regression of different tumours [58,59].

Beside precancerous lesions of non-AIDS-related malignancies, also AIDS-related cancers can be treated successfully with HAART alone. For example, in the treatment of the Kaposi sarcoma of the skin (stage T0), a study achieved an effective treatment with HAART alone in three quarters of the patients [60]. Furthermore, case reports exist indicating the regression of lymphomas under NNRTI-based HAART alone, and in one case, a long-term survival of a patient with small cell lung cancer was described [61–63].

In summary, we could show that EFV has a selective cytotoxic effect on several tumour cell lines. This promotes the idea that EFV may mediate a protective effect against malignancies. In higher concentrations also a therapeutic effect in cancer patients seems possible. Although it could not be proven that EFV acts directly via the cannabinoid receptor, the observed synergistic effect with cannabinoid agonists could indicate an involvement of the cannabinoid system in the antineoplastic effect of EFV.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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