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Review

Cannabinoid receptor ligands as potential anticancer agents – high hopes for new therapies?

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Abstract

Objectives The endocannabinoid system is an endogenous lipid signalling network comprising arachidonic-acid-derived ligands, cannabinoid (CB) receptors, transporters and endocannabinoid degrading enzymes. The CB_1 receptor is predominantly expressed in neurons but is also co-expressed with the CB_2 receptor in peripheral tissues. In recent years, CB receptor ligands, including Δ^9-tetrahydrocannabinol, have been proposed as potential anticancer agents.

Key findings This review critically discusses the pharmacology of CB receptor activation as a novel therapeutic anticancer strategy in terms of ligand selectivity, tissue specificity and potency. Intriguingly, antitumour effects mediated by cannabinoids are not confined to inhibition of cancer cell proliferation; cannabinoids also reduce angiogenesis, cell migration and metastasis, inhibit carcinogenesis and attenuate inflammatory processes. In the last decade several new selective CB_1 and CB_2 receptor agents have been described, but most studies in the area of cancer research have used non-selective CB ligands. Moreover, many of these ligands exert prominent CB receptor-independent pharmacological effects, such as activation of the G-protein-coupled receptor GPR55, peroxisome proliferator-activated receptor gamma and the transient receptor potential vanilloid channels.

Summary The role of the endocannabinoid system in tumourigenesis is still poorly understood and the molecular mechanisms of cannabinoid anticancer action need to be elucidated. The development of CB_2-selective anticancer agents could be advantageous in light of the unwanted central effects exerted by CB_1 receptor ligands. Probably the most interesting question is whether cannabinoids could be useful in chemoprevention or in combination with established chemotherapeutic agents.

Keywords anticancer agent; cancer; cannabinoids; chemoprevention; endocannabinoid system

Introduction

The endocannabinoid system (ECS) comprises the two well-characterised G-protein-coupled receptors (GPCRs) CB_1 and CB_2,[1–3] as well as the putative new GPCRs GPR55 and GPR119,[4,5] former orphan receptors. While cannabinoid binding to GPR55 has been shown (*vide infra*), GPR119 appears to have little affinity for cannabinoids.[6] There is also pharmacological evidence of other putative cannabinoid receptors that remain unknown.[7,8]

The endogenous ligands that activate CB receptors are arachidonic acid derivatives, primarily arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG),[9,10] which are directly released from cell membranes. Endocannabinoids are pleiotropic lipids and their actions are not restricted to cannabinoid receptors. Anandamide also activates transient receptor potential vanilloid 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs), and potentially signals via serotonin 5HT(3) receptors.[11–13] Moreover, 2-AG is an apparently potent ligand for GPR55 whose function is yet to be uncovered.[5,14]

Among other effects, endocannabinoids have been reported to modulate cell differentiation, cell signalling, cell migration and cell fate.[15–17]

The terpenophenolic phytocannabinoids from *Cannabis sativa*, with the prototype cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC),[18] were the first bioactive CB receptor

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ligands to be described and they have served as molecular scaffolds for the chemical development of analogous structures such as CP55,940.[19] The use of CP55,940 as a radioligand has played a key role in the cloning of the CB receptors.

It is probably not an overstatement to claim that without cannabis research the ECS would not have been explored so extensively in the last two decades. It is becoming increasingly clear that the ECS is involved not only in central nervous system regulation (mainly via CB_1) and neuroimmunological processes (both via CB_1 and CB_2) but also in several peripheral physiological processes.[20] It is important to highlight that many of the functions of the ECS that are currently proposed are not yet fully understood. Nonetheless, there is clinical evidence that CB_1 receptor antagonists (inverse agonists) such as rimonabant (SR141716A) are useful in the treatment of obesity and for the improvement of cardiovascular and metabolic risk factors. (Rimonabant was the first therapeutic CB_1 receptor blocker approved in Europe but was withdrawn in October 2008 because of psychiatric side effects.) CB_1 receptor antagonists also have good prospects in other therapeutic areas, including smoking cessation, alcohol addiction and cognitive impairment.[21] CB_1 agonists are useful for the prevention of nausea and vomiting and to stimulate appetite.[22] There is increasing evidence that CB receptors are involved in numerous immune mechanisms and are generally able to attenuate inflammatory processes.[23,24] There are numerous reports on the anti-inflammatory action of CB receptor ligands from animal studies. Positive effects have been reported using CB ligands in models of liver inflammation,[25] neuroinflammation,[26] gut inflammation,[27] skin inflammation[28] and arthritis.[29] Based on CB_2 receptor knockout studies, the involvement of this receptor has been suggested in immune cell function and development, infection, embryonic development, bone loss, liver disorders, pain, autoimmune inflammation, allergic dermatitis, atherosclerosis, apoptosis and chemotaxis.[30] In particular, non-psychoactive CB_2 receptor ligands have been shown to be effective in bone degeneration,[31–34] gut inflammation,[35] neuroprotection[36] and atherosclerosis.[37,38] Given that cannabinoid pharmacology is exceptionally complex, it is difficult to provide a general picture. Overall, however, it seems that CB_2-selective agonists and inverse agonists and CB_1 inverse agonists could be promising therapeutic agents to target chronic inflammatory diseases. Moreover, indirect activation of the ECS via modulation of endocannabinoid tone, such as inhibition of fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase, may be a promising strategy to target pathological inflammatory processes.[39] Different selective and non-selective CB_1/CB_2 receptor ligands have been described, which show potential for a wide range of diseases.[20,21]

Evidence accumulated within the last decade suggests that CB receptor agonists may have antitumour properties in a variety of cancer types; this topic has been reviewed in several cancer-related journals.[40–43] In this review, the recent developments and insights are discussed with respect to CB receptor signalling, ligand selectivity, specificity of effect in different tissues and potential therapeutic relevance.

In 1975, Munson and colleagues reported for the first time that cannabinoids can reduce tumour growth and viability of lung cancer cells *in vitro* as well as *in vivo*.[44] After this initial observation another 20 years passed until more detailed investigations yielded further insights into the anticancer mechanisms of cannabinoids. However, the mechanism of action of CB receptor ligands has started to become uncovered only recently (*vide infra*). While some signalling events involved in the cytotoxic effects exerted by cannabinoids apply for all cellular models, other cellular mechanisms are restricted to only a few cell types. Thus, an important question is how CB receptor activation ultimately leads to inhibition of tumour growth and apoptosis of cancer cells. It is currently not clear whether these effects are specific for certain types of cancer cells, and whether the in-vitro data correlate with more physiological conditions, in particular with regard to the concentrations employed.

CB-receptor mediated signal transduction events leading to anticancer effects

Despite a growing amount of data on the cellular signalling events triggered by cannabinoids in non-neuronal cells, as yet there is no straightforward explanation for the molecular mechanism for their anticancer action. Pharmacological intervention in cancer therapy typically relies on well-defined molecular events that attenuate tumour growth, such as inhibition of microtubule dynamics, inhibition of topoisomerase or DNA intercalation. So far, GPCR signalling in cancer therapy is not a well-designed strategy and there are still many unanswered questions relating to signalling dynamics, limited prolongation of effect due to desensitisation, specificity of signals and potentially unwanted effects. However, with a change of paradigm from the 'one selective drug acting on one target' to network pharmacology,[45] anticancer strategies involving GPCRs may become more interesting in the future. Fundamental to such developments is a better understanding of GPCR cellular signalling cascades, as cancer cells often hijack the normal physiological functions of GPCRs to survive.[46] Targeting of dysregulated kinases in cancer cells could be accomplished via GPCR signalling, and modulation of the cancer kinome may drive tumour cells into apoptosis. As shown in the following sections, CB receptor signalling events that lead to antitumour effects are complex and largely depend on tissue type and physiological context (Figure 1). The major signalling molecules involved in cannabinoid-induced antitumour action are described in the following sections.

Ceramide

Ceramides are composed of sphingosine and fatty acid moieties and are commonly found at high concentrations within the cell membrane, where they are derived from sphingomyelin, one of the major membrane lipids. Ceramide is also a lipid messenger specifically triggered upon activation (e.g. via GPCR activation) and appears to play a key role in the cytoplasm, mediating different effects on cell survival following CB receptor activation.[47] An acute rise of ceramide by sphingomyelin hydrolysis is observed in both glioma cells and normal primary astrocytes after cannabinoid challenge, presumably mediated through the

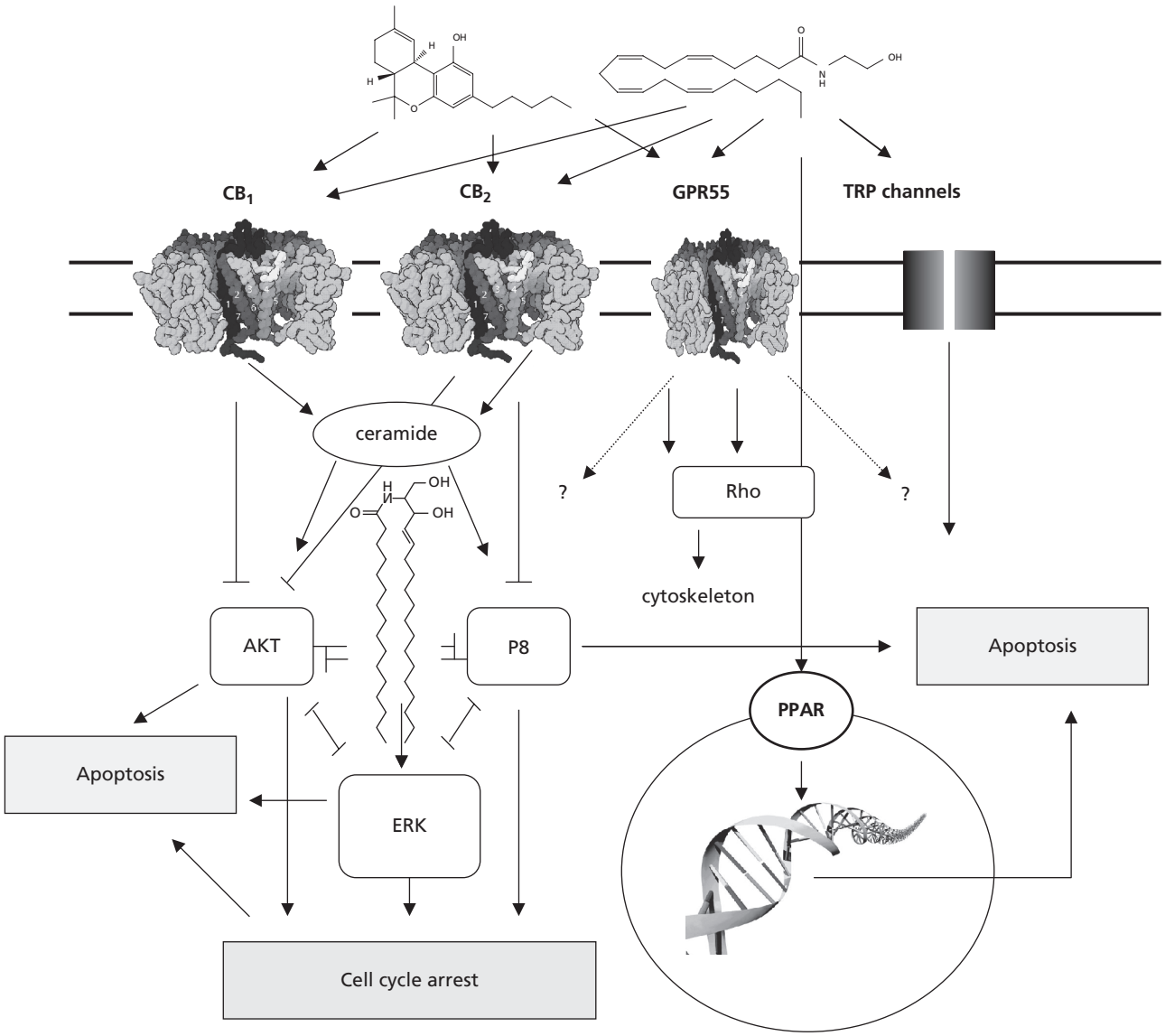

Figure 1 Major signalling pathways involved in the anticancer effects exerted by cannabinoids. The fact that most cannabinoids studied so far interact with more than one receptor adds to the complexity of the pharmacological effects. Ceramide, AKT and ERK are likely to be key mediators in the inhibition of cancer cell growth and induction of apoptosis. AKT, protein kinase B; ERK, extracellular signal-regulated kinase; GPR55, G-protein receptor 55, PPAR, peroxisome proliferator-activated receptor; TRP, transient receptor potential family of channels (including transient receptor potential vanilloid (TRPV)).

CB_1 receptor.[48,49] In addition, malignant glioma cells, as well as other cancer cells (e.g. pancreatic cancer), show sustained de-novo ceramide generation in a CB_1/CB_2-dependent manner, resulting in inhibition of AKT (see below),[50,51] which among other cues will finally drive the cells into apoptosis. Two further studies have shown the role of ceramide as a key player in glioma anticancer action mediated via the CB_2 receptor.[52,53] Interestingly, it was recently shown that either CB_1 or CB_2 receptor activation induces apoptosis through de-novo ceramide synthesis in colon cancer cells, with tumour necrosis factor (TNF)-α acting as a link between CB receptor activation and ceramide production.[54] More strikingly, it appears that cannabinoids are able to protect astrocytes and other neuronal cells from oxidative stress[55] and other neurotoxic signals[56] through ceramide signalling. Where the exact switch for this differential reaction to the apparently identical stimulus lies remains unknown, although in terms of known signalling events it should be concluded that AKT acts downstream of it.

AKT signalling

AKT1 (v-akt murine thymoma viral oncogene cellular homolog), also known as 'AKT' or protein kinase B, represents a group of three enzymes of the serine/threonine-specific protein kinase family and is involved in cellular survival pathways by inhibiting apoptotic processes.[57] Independent of the ceramide-mediated effect on AKT described above, both CB_1 and CB_2 receptors are coupled to

Gi/Go proteins in different cell types and they inhibit adenylate cyclase and can activate phosphatidylinositol 3-kinase (PI3-K), which activates AKT via phosphatidylinositol (3,4,5)-triphosphate (PIP3).[58,59] Along that line, cannabinoid stimulation of whole brain areas and of healthy non-transformed cells such as astrocytes and also of CB_1-positive Chinese hamster ovary cells causes AKT activation almost independently of drug concentration.[60–62] In contrast, one of the general pro-apoptotic signalling events after cannabinoid treatment in cancer cells is the dephosphorylation of AKT1, occurring after both CB_1 and CB_2 receptor activation and probably involving dominant ceramide signalling. PI3-K-dependent AKT activation can be regulated through the tumour suppressor PTEN (phosphatase and tensin homolog), which works essentially as the opposite of PI3-K.[63] PTEN acts as a phosphatase to dephosphorylate PIP3 back to phosphatidylinositol(4,5)P2. This removes the membrane-localisation factor from the AKT signalling pathway. Without this localisation, the rate of AKT activation decreases significantly, as do the all the downstream pathways that depend on AKT for activation. PIP3 can also be dephosphorylated at position 5 by the SH-2-containing inositol phosphatase (SHIP) family of inositol phosphatases, SHIP1 and SHIP2. These polyphosphate inositol phosphatases dephosphorylate PIP3 to form phosphatidylinositol(3,4)P2. An increasing amount of data suggests that AKT inhibition is one of the critical events after cannabinoid administration, determining the cellular downstream effects that ultimately lead to apoptosis (Figure 1). Interestingly, in transformed cancer cells, low nanomolar concentrations of CB agonists lead to AKT phosphorylation through transactivation by epidermal growth factor receptor (EGFR), followed by a prosurvival proliferative burst,[64] whereas higher concentrations decrease the activation status of AKT, usually culminating in growth arrest or apoptosis.[51,65,66] Importantly, overexpression of AKT could rescue cannabinoid-induced apoptosis in melanoma cells, reflecting its essential role in the mediation of cannabinoid-induced apoptosis.[67] Therefore, the concentration range of cannabinoids and the cellular transformation status appear to critically influence the differential cytotoxic effects mediated via AKT signalling. Whether AKT is a molecular switch that determines the often biphasic effects exerted by cannabinoid treatment (*vide infra*) needs to be elucidated.

Extracellular signal-regulated kinase

Another well-known signalling molecule recruited upon treatment of cancer cells with CB receptor agonists is extracellular signal-regulated kinase (ERK). However, reports relating to its activation or inhibition by cannabinoids differ between cancer types, indicating an as yet unclear and maybe more complex role. After incubation with cannabinoids, cells derived from gliomas,[68] prostate cancer[66] and breast cancer[69] display a sustained ERK activation; activation levels of ERK remain unchanged in melanoma cells,[67] whereas in lung[70] and colon cancer cells phosphorylation of ERK was reduced.[65] While inhibition of the usually pro-proliferative signalling molecule ERK is in line with a pro-apoptotic signalling cascade,[71] it is more difficult to understand the contribution of activated ERK to the cannabinoid-induced inhibition of growth. There is evidence that ceramide induced by cannabinoid treatment and inhibition of protein kinase A by Gi-coupled CB receptor stimulation both can cause chronic ERK activation, which is reported to lead to cell cycle arrest and cell death.[68,69] In leukaemia cells, ERK1/2 was induced more strongly by CB_2 receptor-selective agonists than in primary leucocytes.[72] The same study showed that ERK phosphorylation was context dependent, as lipopolysaccharide-induced ERK1/2 activation could be partially blocked by CB_2 ligands.

P8 (Sp(G/C)F-1)

Transcription factor P8 (or candidate of metastasis 1, also referred to as Sp(G/C)F-1), is an endoplasmic-reticulum-associated stress protein able to bind to DNA and is similarly affected by CB-receptor stimulation.[73] After treatment with cannabinoids, it is upregulated in different cancer cell lines, probably in response to de-novo synthesised ceramide, which subsequently leads to co-recruitment of the transcription factors activating transcription factor 4 (ATF4), TRB3 and C/EBP homologous protein (CHOP), all three of which are also critically involved in the cellular response to stress stimuli, probably via both CB_1 and CB_2 receptors.[50,74] P8 seems to be a key factor for cellular sensitivity towards cannabinoids, as siRNA-mediated knock-down of P8 can abolish the cytotoxicity of Δ^9-THC in glioma cells and breast cancer cells. Moreover, P8 is also implicated in the potential synergistic effect of chemotherapeutic agents with cannabinoids.[50,75]

Cell cycle arrest and apoptosis

As classic anticancer agents directly inhibit tumour cell growth, the effects of cannabinoids on cancer cell cycle and apoptosis induction have been investigated in detail. However, no general picture is emerging as with, for example, the G2/M cell cycle arrest typically observed with tubulin-targeting antimitotic agents, probably because different cell types react differently to distinct concentrations of cannabinoids and there are different stress-related mechanisms of action.[42] Moreover, cannabinoids are only moderately cytotoxic and typically exert their effects in the upper-nanomolar and micromolar concentration ranges, depending on the initial cell number and experiment. For example, in U373MG glioma cells expressing CB receptors, Δ^9-THC induced apoptosis at concentrations greater than 5 μM *in vitro*,[76] which appears to be a typical cytotoxic concentration. While some CB-receptor-expressing cancer cells survive treatment with higher micromolar concentrations (e.g. HL60 cells) (Gertsch *et al.*, unpublished data), other cancer cells (e.g. Jurkat T-cells[77]) undergo cell cycle arrest and apoptosis, in part coupled to the signalling pathways described above. Interestingly, R(+)-methanandamide, WIN-55,212-2 and Δ^9-THC lead to up-regulation of tumour suppressor genes such as p16 (INK4A), p27 and p53,[66,78,79] and the oncogene RB is hypophosphorylated,[67,80] which could be due to altered activation levels of either AKT or ERK. Further down this cascade, different cyclins such as D1 and D2, as well as the transcription factor E2F1, are down-regulated, followed by lower activity of cyclin-dependent kinases cdk2, 4 and 6[66] and cdc2,[81] finally causing cell cycle arrest at different cell

cycle check points. These events can either reduce proliferation of cells or prime them for apoptosis. Additionally, cannabinoid challenge with Δ^9-THC and WIN-55,212-2 can prompt cancer cells to undergo apoptosis through the mitochondrial apoptosis pathway, where AKT inhibition classically leads to activation of the pro-apoptotic BCL2-family member BCL2 antagonist of cell death (BAD).[65,82] Upon BAD activation, mitochondrial cytochrome c is released, leading to activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase,[81] which will finally drive cells into apoptosis. This putative mechanism may account for cell cycle arrest and induction of apoptosis in some adherent cells, but the mechanism may be significantly different in other cell types (*vide infra*).

Anti-angiogenesis effects

All of the growth inhibitory mechanisms of cannabinoids discussed so far are direct cellular effects. However, cannabinoids can modulate intercellular signalling, leading to modulation of important regulatory factors involved in inflammation and cellular activation and thereby influence tumour development indirectly. In this regard, the effect of cannabinoid treatment on tumour angiogenesis ranks most prominently.[83] Different cannabinoid compounds with varying CB_1 and CB_2 receptor affinities decrease the formation of new blood vessels in tumours of different origins (e.g. non-melanoma skin cancer and glioma) by downregulating essential proangiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor and angiotensin II accompanied by dephosphorylation of VEGF receptors 1 and 2.[79,84,85] These events could also partially account for a lower rate of metastasis, as this process is crucially linked to peri- and intratumoural vascularisation.[86]

It is not only cancer cells that are influenced by cannabinoids; endothelial cell sprouting and vessel formation were blocked in various angiogenesis assays by application of R(+)-methanandamide.[87] Both the CB_1 receptor agonist ACEA and the CB_2 receptor agonist JWH-015 decreased the weight and vascularisation of carrageenan-induced granulomas in rats and reduced mast cell number and activation in granulomatous tissue.[88] Interestingly, in this study ACEA and JWH-015 prevented the transcription and expression of rMCP-5, a protein involved in sprouting and advance of new blood vessels. Currently, it is unclear whether these effects are actually mediated via CB receptor signalling or whether other as-yet unknown mechanisms are involved. However, the effectiveness of the CB_2-receptor-selective agonists JWH-015 and JWH-133 in blocking monocyte migration[89] suggests that CB_2 activation may generally inhibit cell migration and vessel formation. In fact, cell migration is a key event in tumour metastasis and angiogenesis. Various studies have shown that cannabinoids affect cell migration through both CB_1 and CB_2 receptors, as well as through mechanisms related to elusive cannabinoid targets.[17,90] Overall, the evolving picture is rather complex, as apparently anandamide and 2-AG produce opposite effects and only 2-AG signals via CB receptors.[17] Endocannabinoids now join the list of factors involved in bone marrow cell proliferation and differentiation, the ECS being a part of a highly complex lipid network that is still poorly understood.

The CB_2 receptor – a proto-oncogene?

It has been shown that cannabinoids stimulate proliferation of neural stem/precursor cells acting via both CB_1 and CB_2 receptors, leading to activation of the PI3-K/AKT pathway.[91] Both the anti- and pro-proliferative effects exerted via CB receptor activation are intriguing and clearly deserve further investigation. In leukaemia cells, the CB_2 receptor has been suggested to act as a proto-oncogene, which under certain circumstances may turn into an oncogene that promotes carcinogenesis. Valk and colleagues have identified the new common virus integration site Evi11 and demonstrated that the gene encoding the CB_2 receptor (*Cnr2*) is its potential target, thus suggesting that *Cnr2* could be a proto-oncogene.[92] Subsequent research by the same group demonstrated that the CB_2 receptor can act as an oncoprotein that blocks neutrophilic differentiation when overexpressed in myeloid precursor cells and that haematopoietic precursor cells expressing high levels of CB_2 have increased susceptibility for leukaemia development, thus suggesting that CB_2 and Evi1/Evi11 might collaborate in leukemogenesis.[93] Moreover, the CB_2 receptor appears to mediate this activity through mitogen-activated protein kinase ERK/(MEK) and PI3-K pathways.[94] This would suggest that blocking rather than activating the CB_2 receptor should be beneficial in the treatment of leukaemia. Alternatively, stabilising the CB_2 receptor in its inactive state using inverse agonists could counteract leukemogenesis. High CB_2 receptor expression in myeloid precursors is also associated with different immunomodulatory effects, such as inhibition of immune cell migration[90] and inhibition of TNF-α expression.[20,24] Since both CB_2-selective agonists and inverse agonists are anti-inflammatory *in vivo* and, paradoxically, apparently both via CB_2 interaction,[20,24,95,96] it is currently not clear whether CB_2-selective agonists or inverse agonists should be developed for therapeutic intervention. Moreover, the effects of these ligands may differ substantially *in vitro* and *in vivo*, and an agonist *in vitro* may act as an antagonist *in vivo* and vice versa.[96] However, it is still not clear whether the CB_2 receptor actually becomes an oncoprotein and whether this relates to mutations or increased receptor expression. On the other hand, it is already clear that the CB_2 receptor regulates cell growth and differentiation in promyelotic human cells, being a regulator of signal transduction via the oncogenic Erk1/2 pathways and execution of mitogenic signals that are relevant to cell differentiation (*vide supra*). In light of the fact that healthy humans have a high surface expression of functional CB_2 receptors in monocytes and B cells but a low surface expression in T cells (Gertsch *et al.*, unpublished data), it is possible that *Cnr2* is a proto-oncogene in T cells but not in promyelocytic cells (precursors of monocytes/macrophages and dendritic cells). This would be confirmed by the fact that cannabinoids induce apoptosis in Jurkat T cells but not HL60 cells. Another factor that complicates studies with the CB_2 receptor is that *Cnr2* has undergone more rapid evolution than *Cnr1*, leading to pronounced species differences in ligand–receptor interactions (receptor affinities and G-protein recruiting)[97] and therefore efficacy

in animal studies cannot always be extrapolated to humans. There are still too many uncertainties to draw any conclusion regarding cannabinoid treatment of lymphomas, despite studies showing overexpression of the CB_2 receptor.

Intriguingly, CB_1 agonists can induce apoptosis in mantle cell lymphoma via CB_1 receptor activation,[98] although this was under conditions where the CB_2 receptor is also activated, as non-selective cannabinoids were used in this study (Table 1; Figure 2). Moreover, CB_2 receptor activation leads to anticancer effects in other tissues. CB_2 receptor overexpression may contribute to the regression of human anaplastic thyroid tumour in nude mice following interleukin-12 gene transfer,[139] suggesting an inhibitory role of CB_2 in thyroid carcinogenesis. *In vitro*, Met-F-AEA was shown to reduce thyroid tumour growth[79] and to induce apoptosis in thyroid carcinoma cells.[140] Interestingly, 3,3′ diindolmethane (DIM), which is an anticarcinogenic metabolite generated by ingestion of indole-3-carbinol commonly found in *Brassica* vegetables, has recently been shown to be a weak partial CB_2 receptor agonist.[141] However, DIM has other targets that may explain its anticancer effects, including elastase.[142] Nonetheless, it is tempting to speculate that dietary CB_2 ligands such as beta-caryophyllene[72] and DIM may exert potentially chemopreventive effects and inhibit carcinogenesis via CB_2 receptor interaction.

Anticancer effects of cannabinoids independent of CB receptors

Not all effects of cannabinoids are mediated through classic CB receptors, and there is an increasing amount of data showing that many ligands are not specific for CB receptors. Often well-designed protein-selective ligands are specific for a certain target until their non-specificity is shown, rendering erroneous initial conclusions drawn from pharmacological experiments. This may also be true for CB receptor inverse agonists (i.e. antagonists). Recently, it was shown that the CB_1 selective agonist SR141716A (rimonabant) also binds to GPR55[143] and can act as GPR55 receptor antagonist.[144] A recent report using a beta-arrestin Pathhunter assay showed that SR141716A and AM251 induced significant effects via GPR55, while endocannabinoids were only weakly active.[141] While the number of CB_1- and CB_2-selective agonists and antagonists is increasing, it is not clear whether these compounds exert other cellular actions at the often high concentrations/doses used (Table 1). Moreover, studies like the one performed on the murine lymphomas L-4, LSA and P815 with Δ^9-THC treatment may be hard to interpret, as Δ^9-THC apparently more strongly activates GPR55 than CB receptors,[143] the role of which in cancer remains to be elucidated. Recently, it was shown that GPR55 signals via Rho and activates nuclear factor of activated T cells (NF-AT).[145] Because NF-AT signalling plays a potential role in cancer growth (e.g. in Burkitt's lymphoma),[146] this certainly complicates the interpretation of studies performed with cannabinoids without using knockout mice as controls. In fact, the new cannabinoid-like receptor GPR55 with signalling distinct from CB_1 and CB_2 may be a hitherto neglected receptor with regard to the anticancer effects of several cannabinoids. Many cannabinoids interact with GPR55 and the receptor appears to be present in numerous cell types.[4,14,143] GPR55 is activated by a whole range of plant, synthetic and endogenous cannabinoids and is blocked by cannabidiol, a non-psychoactive phytocannabinoid,[5,143] and SR141716A.[144] Cannabidiol induces a concentration-dependent increase in FAAH activity and 5-lipoxygenase activity in U87 glioma cells, reducing the growth rate.[147] The most striking difference reported so far is the agonist activity of the CB_1 receptor antagonists AM251 and AM281 at GPR55,[5,141] rendering elucidative studies of CB receptor specificities a challenging task. To date, nothing has been published about its expression in cancer cells and therefore putative effects of cannabinoids through GPR55 cannot be ruled out and deserve further attention. Activation of GPR55 by AM251[125] could also explain the observation that this drug exerts antiproliferative effects on pancreatic cancer cells in the low micromolar range. Similarly, another article reported cell cycle arrest in breast cancer cells treated with the CB_1 antagonist SR141716A,[148] an effect that could, at least in part, also be mediated by interaction with GPR55.

TRPV channels, PPARγ and 5HT(3) receptor are also non-cannabinoid targets of cannabinoids.[13,149,150] Anandamide and similar cannabinoid structures activate the vanilloid receptor (VR1 or TRPV1), which can be blocked with the TRPV1 antagonist capaszepine. In cervical cancer cells with aberrant TRPV1 expression, the stimulation of TRPV1 rather than CB_1 or CB_2 receptors accounts for the apoptosis-inducing effects of anandamide,[101] whereas the migration-reducing effects of R(+)-methanandamide could be blocked by antagonists to TRPV1, CB_1 and CB_2, highlighting the complexity of cannabinoid-evoked signal generation.[151] Recently, De Petrocellis and colleagues suggested that phytocannabinoids and cannabis extracts exert some of their pharmacological actions by interacting with TRPA1 and TRPM8 channels, with potential implications for the treatment of pain and cancer.[152]

Another well-described mechanism for CB-independent action of certain CB receptor agonists is their binding to some members of the nuclear receptor transcription factor superfamily PPARs,[12,150] although the extent to which this mechanism is involved in the effects of cannabinoids as anti-tumour agents remains poorly described. Intriguingly, in the HepG2 hepatoma cell line, PPARγ may play a key role in WIN 55,212-2-induced apoptosis.[138] The non-psychoactive Δ^9-THC analogue ajulemic acid, which binds to the CB_2 receptor, has been shown to exert antitumour effects in glioma cells.[107] Interestingly, ajulemic acid is also an activator of PPARγ.[153]

In hormone-dependent breast and prostate carcinoma, cannabinoid treatment can decrease expression levels of receptors involved in their pro-proliferative response to the cytokines prolactin, nerve growth factor (NGF) and androgen.[69,129,154] Namely, in prolactin-dependent breast cancer, 2-AG and anandamide downregulate the prolactin receptor and the trkNGF receptor, whereas in postate cancer, 2-AG, anandamide and WIN-55,212-2 reduce levels of prolactin and androgen receptors. In addition to these indirect proliferation-inhibiting effects, certain plant-derived and synthetic cannabinoids inhibit the multidrug-transporter ABCG2 and p-glycoprotein in mouse embryonic fibroblasts (MEF), immortalised renal cells, Caco-2 cells and rat brain microvessel cells,

Table 1 Effects of cannabinoid receptor ligands on cancer cells *in vitro* and *in vivo*

Cancer	Cell lines	Effects	Compound	Reversible by CB_1/CB_2 antagonists?	*In vivo*?	Reference
Glioblastoma multiforme	U251-MG and U87-MG	Cell cycle inhibition	Δ9-THC	?	no	Galanti *et al.* 2007[78]
	SF126, U87, U251, U373-MG, SF188, GBM primary cells	Cell death, reduced proliferation	WIN-55,212-2, Δ9-THC	SR1, SR2	no	McAllister *et al.* 2005[99]
Glioma	U87	ROS, apoptosis	Cannabidiol	no	yes	Massi *et al.* 2006[100]
	C6, U87mg	Apoptosis	Δ9-THC	SR1, SR2	yes	Carracedo *et al.* 2006[50]
	?	Apoptosis	AEA	no?	no	Contassot *et al.* 2004[101]
	C6	Cell death by oxidative stress	Δ9-THC	SR1	no	Goncharov *et al.* 2005[102]
	C6	Apoptosis	WIN-55,212-2	no	no	Ellert-Miklaszewska *et al.* 2005[82]
	U87, U373	Anti-proliferative	Cannabidiol	SR2	no	Massi *et al.* 2004[103]
	H4 neuroglioma cells	Apoptosis	R-methanandamide	no	no	Hinz *et al.* 2004[104]
	C6	Anti-proliferative	1-AG, phosphate esters of anandamide	no	no	Fowler *et al.* 2003[105]
	C6	Apoptosis	Δ9-THC	no	no	Gomez del Pulgar *et al.* 2002[51]
	C6	Anti-proliferative	AEA, 2-AG, JWH-015, CP55,940	SR1/2, AM251, AM630, capsazepine	no	Jacobsson *et al.* 2001[106]
	C6, U87mg	Reduced viability	Ajulemic acid	SR2	yes	Recht *et al.* 2001[107]
	C6	Apoptosis	Δ9-THC	SR1 & SR2 combination	yes	Galve-Roperh *et al.* 2000[68]
	C6	Apoptosis	Δ9-THC	SR1	no	Sanchez *et al.* 1998[48]
	C6	Apoptosis	JWH-33	SR2	yes	Sanchez *et al.* 2001[108]
	C6	Inhibition of invasion, down-regulation of MMP-2	Δ9-THC, JWH-133	no	yes	Blazquez *et al.* 2008[52]
	U87-mg, T98G, LN-229, MT310	Reduced viability	C1′-phenyl-substituted Δ(8)-THC	no	no	Krishnamurthy *et al.* 2008[109]
Neuroblastoma	B102	Growth-inhibition	Δ9-THC	no	no	Cabral *et al.* 1987[110]
	NB2A, (C6)	Anti-proliferative	Δ9-THC	no	no	End *et al.* 1977[111]
Leukaemia	Jurkat	Apoptosis	Δ9-THC	SR1 & SR2	no	Jia *et al.* 2006[77]
	Jurkat, MOLT-4	Apoptosis	Cannabidiol	SR2	yes	McKallip *et al.* 2006[112]
	Jurkat	Apoptosis	Δ9-THC	SR2	no	Herrera *et al.* 2005[113]
	Jurkat	Apoptosis	Δ9-THC	no	no	Lombard *et al.* 2005[114]
	EL-4, LSA, P815, Jurkat, Molt4	Apoptosis	Δ9-THC, HU210, anandamide, JWH-015	SR2	yes	McKallip *et al.* 2002[115]
	K562	Anti-proliferative	Δ9-THC	no	no	Dvilansky *et al.* 1984[116]
	L1210 murine leukemia	Anti-proliferative	Δ8-THC	no	yes	Tucker & Friedman 1977[117]
	L1210 murine leukemia, Lewis lung carcinoma	Inhibition of DNA synthesis	Δ9-THC, Δ8-THC	no	yes	Carchman *et al.* 1976[118]
Lymphoma	Rec1, Jeko, JVM-2	Apoptosis	WIN-55,212-2, R(+)-methanandamide	SR1 & SR2	no	Gustafsson *et al.* 2006[98]
	MCL cells from biopsies	Apoptosis	WIN-55,212-2, anandamide	no	no	Flygare *et al.* 2005[119]
	Rec-1, MEL1, MEL2, Raji, Namalwa, JEKO-1	Apoptosis	R(+)-methandandamide	SR1 & SR2	yes	Gustafsson *et al.* 2008[120]
Lung cancer	A549 and SW-1573	Growth inhibition, anti-angiogenic, anti-migration	Δ9-THC	no	yes	Preet *et al.* 2008[70]

(Continued)

Table 1 *(Continued)*

Cancer	Cell lines	Effects	Compound	Reversible by CB_1/CB_2 antagonists?	*In vivo*?	Reference
	Lewis lung carcinoma	Anti-proliferative	Δ9-THC, Δ8-THC, cannabidiol	no	no	Friedmann 1977[121]
	Lewis lung adenocarcinoma cells	Anti-proliferative	Δ9-THC	no	no	White *et al.* 1976[122]
	L1210 murine leukemia, Lewis lung carcinoma	Inhibition of DNA synthesis	Δ9-THC, Δ8-THC	no	yes	Carchman *et al.* 1976[118]
	Lewis lung adenocarcinoma cells	Reduced tumour growth	Δ9-THC, Δ8-THC, cannabinol	no	yes	Munson *et al.* 1975[44]
Colon cancer	SW480, HCT15	Apoptosis	Δ9-THC	AM251	no	Greenhough *et al.* 2007[65]
	?	Cell death	Anandamide	no	no	Patsos *et al.* 2005[123]
	CaCo-2, DLD-1	Anti-proliferative	2-AG, anandamide, HU210	SR1 & SR2	no	Ligresti *et al.* 2003[124]
	DLD-1, HT29, primary human tumor	Apoptosis via ceramide	ACEA, CB13	no	no	Cianchi *et al.* 2008[54]
	CaCo-2	Anti-proliferative, reduced viability	HU-210, anandamide, NAGly	no	no	Gustafsson *et al.* 2008[120]
Pancreatic cancer	MiaPaCa, Panc-1	Apoptosis	Δ9-THC, WIN-55,212-2, JWH-133	SR2	yes	Carracedo *et al.* 2006[74]
	MiaPaCa2	Apoptosis	ACEA, AM251, JWH-015, AM630	no	no	Fogli *et al.* 2006[125]
Breast cancer	MDA-MB-231, T47D and MCF-7	Cell cycle arrest, lipid rafts critically involved	SR141716	no	no	Sarnataro *et al.* 2006[126]
	EVSA-T, MCF-7, MDA-MB-468, MDA-MB-231, SKBr3, T47D	Cell cycle block, apoptosis	Δ9-THC	SR1, better SR2	no	Caffarel *et al.* 2006[84]
	HBcc	Anti-proliferative	Anandamide	no	no	De Petrocellis *et al.* 2002[127]
	HBcc	Anti-proliferative	PEA + anandamide	SR2	no	Di Marzo *et al.* 2001[128]
	HBCC, DU145	Anti-proliferative, PRL/NGF receptors↓	Anandamide, 2-AG, HU210	SR1	no	Melck *et al.* 2000[129]
	MCF-7	Anti-proliferative, PRL/NGF receptors↓	Anandamide	SR1	no	Melck *et al.* 1999[69]
	EFM-19	Anti-proliferative	Anandamide	SR1	no	Bisogno *et al.* 1998[130]
	EFM-19, MCF7	Anti-proliferative	Anandamide, 2-AG, HU210	SR1	no	De Petrocellis *et al.* 1998[131]
Prostate cancer	LNCaP	Apoptosis	WIN-55,212-2	SR1 SR2	yes	Sarfaraz *et al.* 2006[66]
	DU145, LNCaP, PC-3	Apoptosis	WIN-55,212-2	SR1, SR2	yes	Sarfaraz *et al.* 2005[132]
	DU145, LNCaP, PC-4	Cell death	Anandamide	SR1	no	Mimeault *et al.* 2003[133]
	PC-3	Cell death	Anandamide	no	no	Sarker *et al.* 2003[134]
	HBCC, DU145	Anti-proliferative, PRL/NGF receptors↓	Anandamide, 2-AG, HU210	SR1	no	Melck *et al.* 2000[129]
	PC3	Apoptosis	Δ9-THC	no	no	Ruiz *et al.* 1999[135]
Cervix carcinoma	CC299, Caski, HeLa	Apoptosis	Anandamide	VR1 inhbition	no	Contassot *et al.* 2004[101]
Melanoma	B16, A375	Apoptosis, anti-angiogenic	WIN-55,212-2, Δ9-THC, JWH-133	SR2	yes	Blazquez *et al.* 2006[67]
Skin tumour	PDV.C57, HaCa4	Apoptosis, anti-angiogenic	WIN-55,212-2, JWH-133, Δ9-THC	SR1 & SR2	yes	Casanova *et al.* 2003[85]
Thyroid cancer	KiMol, TK-6	Growth inhibition	Met-F-anandamide	SR1	yes	Portella *et al.* 2003[79]
Thymoma	?	ROS, apoptosis	Cannabidiol	no	no	Lee *et al.* 2008[136]
Cholangiocarcinoma	Mz-ChA-1, HuH28, HuCC-T1, SG231	Apoptosis (FAS to lipid raft)	Anandamide	no	no	DeMorrow *et al.* 2007[137]
Hepatoma	HepG2	Apoptosis	WIN-55,212-2	GW9662 & T00709007 (PPARγ)	no	Giuliano *et al.* 2009[138]

GBM, glioblastoma multiforme; NGF, nerve growth factor; PPAR, peroxisome proliferator-activated receptor; PRL, prolactin; ROS, reactive oxygen species; SR1, SR141716A (selective CB_1 receptor antagonist/inverse agonist); SR2, SR144528 (selective CB_2 receptor antagonist/inverse agonist); THC, tetrahydrocannabinol.

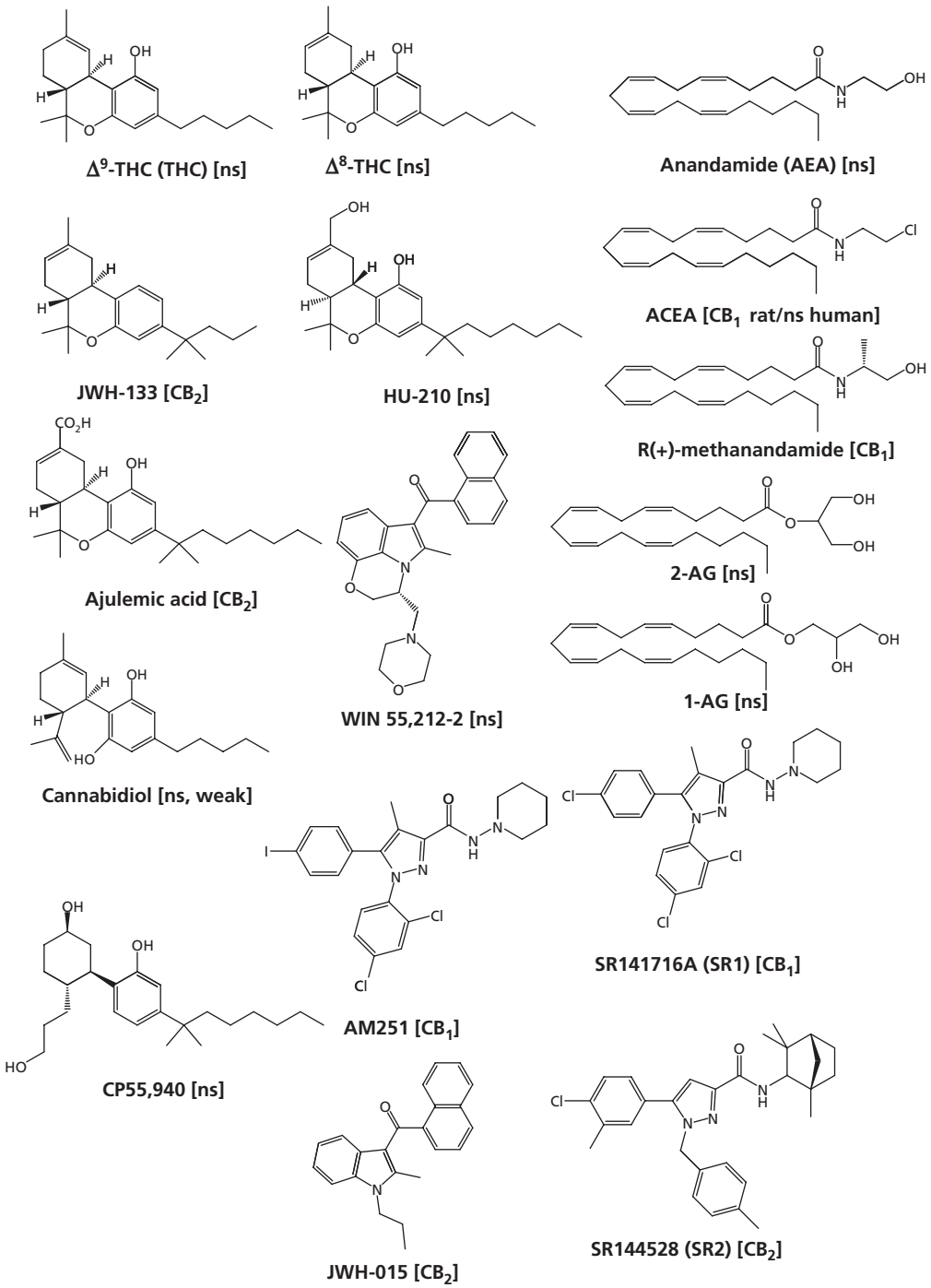

Figure 2 Cannabinoid receptor ligands commonly used in the study of the anticancer effects. The binding selectivity towards CB_1 and CB_2 receptors is indicated in square brackets. ns, non-selective; THC, tetrahydrocannabinol.

which potentially contribute to sensitisation to and accumulation of chemotherapeutic agents such as topotecan and doxorubicin.[155–159]

Is elevation of endocannabinoid levels in tumours good or bad?

There is good evidence that certain tumour cells overexpress endocannabinoids, which are typically released during cellular stress (e.g. upon activation of Toll-like receptor pathways). For example, in colon tissue anandamide levels are significantly upregulated after malignant transformation.[160] Since endocannabinoids activate both CB_1 and CB_2 receptors, they could initiate the anticancer signalling pathways described above. This leads to the obvious question of why a growing tumour should kill itself by such a mechanism? It has previously been postulated that endocannabinoid tone may be

a means of controlling endogenous tumour growth (reviewed by Flygare & Sander[42]). However, despite data from several studies showing anticancer effects mediated by the endogenous ligands anandamide and 2-AG (either directly or by increasing their levels by blocking degradation or transport), it is still not clear whether the ECS is an endogenous anticancer or a procarcinogenic system. The latter was proposed in a study using knockout mice, in which CB_1/CB_2 receptors were suggested to play a positive role in UV-induced inflammation and development of skin cancer; the study showed that in the skin UVB activates nuclear factor κB via CB receptors, leading to increased TNF-α expression.[161] As cannabinoids at low concentrations typically inhibit TNF-α expression from immune cells,[20,24] this seems to be rather contradictory. However, several lines of evidence suggest that the ECS in the skin is different from the ECS in the rest of the body, and that CB_2 receptor agonists seem to be pro-inflammatory in the skin.[20,28] Somewhat contradictorily, CB receptor activation in melanoma has been shown to reduce tumour growth via AKT signalling.[67] A report by Aguado and colleagues on glioma stem-like cells showed that cannabinoids such as HU210 and JWH-133 cause higher expression of glial differentiation markers in a CB_1 and CB_2-dependent manner, respectively.[162] Upon engraftment of these more differentiated cells into mice, a lower rate of gliomagenesis was observed than in engrafted control cells, suggesting a potential inhibitory role for CB receptor agonists in cancer stem cell differentiation. The CB_2 receptor agonists JWH133 and Δ^9-THC were able to inhibit glioma cell invasion in mice, probably due to down-regulation of metalloproteinase-2 expression.[52] A recent study suggests that high CB_1 receptor expression is associated with severity of prostate cancer and outcome.[163] Obviously, the role of the ECS system is not clear, and it is likely that different tissues employ the ECS differently. While for many tissues (central nervous system, liver, gut, arteries, etc.) it may be beneficial to activate the ECS, other tissues may develop pathologies (adipose tissue, skin). As pointed out in a recent review by Di Marzo,[20] endocannabinoids may be able to act in opposite directions depending on the physiological context. Furthermore, physiological processes are dynamic whereas experiments often look at single time points rather than the overall kinetics. This makes the development of new cannabinoid therapeutics a challenging task. With regard to cancer, it needs to be emphasised that CB receptor expression in cancer cells has largely been determined at the level of mRNA expression and by Western blots,[67,120,154] which does not allow for the fact that surface expression may vary and may not correlate with gene expression. Unpublished data from our laboratory clearly indicate that many cancer cells lack CB surface expression despite being positive in RT-PCR and Western blot analyses. Thus, studies ignoring the fact that CB receptors are probably not coupled to G-proteins in many cancer cell lines may lead to potentially erroneous conclusions.

In spite of the vast number of publications supporting the use of cannabinoids as anticancer agents, it should be noted that there are some potential not insignificant drawbacks, such as the apparently prosurvival effects of cannabinoids at low concentrations in cancer cells and their potential immunosuppressive action (*vide supra*). Apparently, nanomolar concentrations of Δ^9-THC, comparable with those detected in the serum of patients after administration of Δ^9-THC, accelerate proliferation of cancer cells instead of inducing apoptosis.[64] The same observation also holds true for the in-vitro incubation of several cancer cell types with WIN55,212-2 and HU210, an effect that was attributed to transactivation of the EGFR, leading to activation of the AKT and MAPK signalling pathways.[64] In this regard, the use of cannabis as it is already approved as an adjuvant to chemotherapeutic treatment regimens[164,165] could potentially boost tumour growth, although clinical evidence for this hypothesis is lacking.

The second critical point relates to the fact that Δ^9-THC potentially alters the immune status by suppressing the cell-mediated TH_1 response, which is of particular relevance in the battle against tumour cells.[166] Since TNF-α expression is typically inhibited by low cannabinoid concentrations and TNF-α itself inhibits tumour growth,[167] it is not clear what the effect of cannabinoids on physiological tumour development is. On the other hand, a pro-inflammatory environment can lead to carcinogenesis[167,168] and cannabinoids may be able to prevent this. Two recent studies have shown that loss of CB_1 led to an increase in carcinogenesis in colon cancer[169] and enhanced endocannabinoid tone prevented colon cancer,[170] thus pointing to a suppression of colon carcinogenesis by the ECS and CB_1 receptor. Moreover, the CB_2 receptor has been suggested to exert beneficial regulatory effects in the gut, such as attenuation of inflammation and probably colon cancer.[35] With the commonly used xenograft animal models, where human cancer cells are grafted into immunodeficient mice, it is impossible to predict the impact of the cannabinoid treatment on the immune surveillance of the tumour; data from a melanoma allograft model suggest that the inhibitory effects on tumour growth and formation may be independent of immune status of the mice and site of drug injection.[67]

Conclusions and outlook

Cannabinoids may have anticancer effects in the appropriate context but their effects may not be sufficiently radical for chemotherapy. Currently, Δ^9-THC (Marinol) and the synthetic derivative Nabilone are successfully used as adjuvants to chemotherapeutic treatment because they prevent nausea and vomiting and stimulate appetite.[22,165] Moreover, the Δ^9-THC- and cannabidiol-containing *C. sativa* extract Sativex is used for the symptomatic relief of neuropathic pain in adults with multiple sclerosis and as an adjunctive analgesic treatment for adults with advanced cancer.[164,171] Currently, there are no clinical data indicating that co-treatment with these cannabinoids improves or reduces the anticancer efficacy of the actual chemotherapeutic agents. Such clinical comparisons would be very interesting. Based on current knowledge, the ECS may be a system that, under the appropriate conditions, produces synergy with established chemotherapeutic agents. *In vitro*, subcytotoxic concentrations of Δ^9-THC were shown to sensitise leukaemia cells to chemotherapeutic agents such as doxorubicin and vincristine.[172] Several other reports have dealt with the potential synergism of cannabinoids with chemotherapeutic

agents such as topotecan and doxorubicin.[50,75,155–159] Clearly, more research should be directed towards the potential synergism and antagonism of cannabinoids in chemotherapy. Despite several promising reports from studies with cannabinoids in animal xenograft models, data relating to humans are limited and therapeutic benefits therefore remain speculative. Moreover, there are numerous apparently non-toxic natural products that potentially exert chemopreventive or antitumour effects, many of which have been confirmed in animal models,[173] but few have been tested in a clinical setting. This is largely because of limited financial resources and the high risk for a pharmaceutical company to become involved in clinical studies with known natural products in general and cannabinoids in particular.

While data obtained in different cellular and animal models suggest that cannabinoid ligands could be useful to treat certain forms of cancer, the abundance of CB receptors in different tissues could clearly be a problem with regard to potentially unwanted effects. However, based on a recent review,[174] cannabinoids appear to be selective antitumour agents that kill glioma cells without affecting the viability of non-transformed counterparts. Intriguingly, they mention a pilot clinical trial on patients with glioblastoma multiforme which showed remarkable antitumour effects of cannabinoids and a good safety profile, thus setting the basis for further studies.

Interestingly and somewhat surprisingly, most studies to date have been carried out with ligands that target both CB_1 and CB_2 receptors in a non-selective manner. In terms of a potential therapeutic application the unwanted psychotropic effects mediated via CB_1 could be problematic. However, there is still a limited amount of data on CB_2-receptor-selective anticancer effects (by agonists, silent antagonists or inverse agonists) and the potential therapeutic relevance remains unclear. Given that the CB_2 receptor mediates several of the effects reported for CB_1 without being psychotropic, more research should be directed to the role of CB_2 in cancer. Future studies using CB_1- and CB_2-selective ligands in combination with animal models in which CB receptors have been genetically deleted should be useful. Importantly, conclusions drawn from experiments with CB receptor antagonists may be misleading because these ligands potentially interact with other targets, such as the CB_1 receptor inverse agonists (antagonists) SR141716A and AM251 with GPR55 (*vide supra*).

Maybe more promising is the potential of the ECS in the suppression of cancer development. It is tempting to speculate that the ECS is involved in carcinogenesis and tumourigenesis in certain tissues, as it potentially modulates the biochemical microenvironment, probably leading to modulation of cytokines and growth factors. To provide stronger evidence, future research will have to uncover potential ways of chemoprevention by cannabinoids. Given that the ECS regulates immune processes, it is tempting to believe that the ECS can directly affect carcinogenesis by modulating inflammatory stress that leads to carcinogenesis. More than 10 years ago, Sidney and colleagues[175] concluded that not only is the evidence linking cannabis smoking to cancer negative, but the largest human studies cited indicated that cannabis users had lower rates of cancer than non-users. Moreover, those who smoked both cannabis and tobacco had lower rates of lung cancer than those who smoked only tobacco – a strong indication of chemoprevention. However, this statement was recently challenged by a study performed by Aldington and colleagues,[176] which showed that cannabis smoking increased the risk for lung cancer, but it is not certain that cannabinoids are responsible for this correlation.[177] Along the same lines, it is not clear whether chronic marijuana use is correlated with an increased incidence of testicular germ cell tumours[178] because of cannabinoid action. An increasing amount of data shows attenuation of tumour growth by both orally and locally administered cannabinoids in different animal models, raising high hopes for potentially new treatments, in particular in combination with established chemotherapeutic agents (*vide supra*). Future research along that line will have to show whether cannabinoids or cannabimimetic agents may reduce tumour growth *in vivo* synergistically when used with chemotherapeutic agents. Meanwhile, the exact mode of action of cannabinoids, the role of classic CB receptors, and the potential involvement of GPR55 and the other non-cannabinoid targets remain to be elucidated. In conclusion, a better understanding of the underlying physiological processes of the ECS in malignancy is needed before anticancer agents that act via the ECS can be developed (Figure 3).

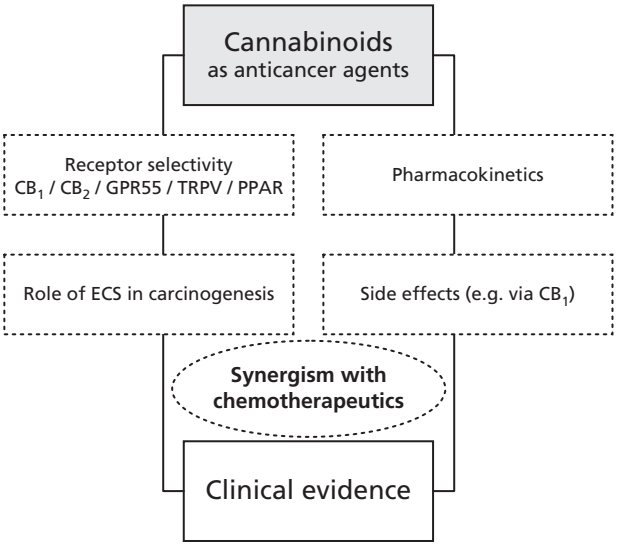

Figure 3 Important issues that need to be addressed (shown in dotted boxes) prior to the development of cannabinoid-type anticancer agents in a clinical setup. ECS, endocannabinoid system; GPR55, G-protein receptor 55; PPAR, peroxisome proliferator-activated receptor; TRPV, transient receptor potential vanilloid.

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