

REVIEW

Combining naturally occurring polyphenols with TNF-Related Apoptosis-Inducing Ligand (TRAIL): a promising approach to kill resistant cancer cells?

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Running title: TRAIL and polyphenols in anticancer therapy.

Abstract

TRAIL and its receptors are attractive targets for anticancer therapy owing to their ability to trigger apoptosis selectively in cancer cells but not normal cells. To date, many combinatorial strategies, such as chemotherapy or radiotherapy, have given encouraging results for overcoming TRAIL resistance in preclinical models. In this review, we provide an overview of the molecular mechanisms underlying sensitization to TRAIL-induced apoptosis by polyphenols. These naturally occurring compounds can restore tumor cell sensitivity to TRAIL-induced cell death with no apparent toxicity towards normal cells. Both extrinsic and intrinsic pathways can be modulated by polyphenols, the activation of which largely depends on the cell type, the particular polyphenolic compound, and the conditions of treatment. The large variety of polyphenol cellular targets could prove useful in circumventing TRAIL resistance. The relevance of these combined treatments for cancer therapy is discussed in the light of recent preclinical studies.

Keywords Cancer, Apoptosis, TRAIL, DR4, DR5, Polyphenol, Flavonoid, Stilbene.

Introduction

During the last decade, the cytokine TRAIL (APO2-L or TNF-related apoptosis-inducing ligand) and agonistic antibodies targeting TRAIL receptors have gained considerable interest

in cancer therapy, due to their ability to induce tumor regression in preclinical studies with no significant side effects [1]. However, it appears that often treatment with TRAIL or agonistic antibody alone is not sufficient for an effective apoptotic response. Mapatumumab, an antibody which targets TRAIL-R1, exhibits little clinical activity as single agent in patients with refractory colorectal cancer [2]. Other clinical trials nevertheless provide encouraging results when recombinant human TRAIL or anti-TRAIL-R1 or -R2 agonistic antibodies are combined with conventional chemotherapy [3]. The problem in current cancer therapy is the occurrence of a few resistant tumor cells that cause cancer relapse. The current challenge in oncology is therefore to find a treatment able to eradicate the tumor without triggering resistance, and to limit as much as possible its toxicity against normal cells. Combined treatments seem to be the best way to reach this objective. With this in mind, since 2002 it progressively became apparent that the combination of TRAIL with naturally occurring polyphenols would represent an attractive therapeutic approach (Table 1).

TRAIL and its receptors in cancer therapy

TRAIL is a member of the Tumor Necrosis Factor (TNF) gene superfamily that displays great apoptosis-inducing activity against cancer cells both *in vitro* and *in vivo*. Unlike FasL or TNF, which are known to cause severe toxicity to liver tissue, TRAIL was shown to be safe following *in vivo* administration [4-6]. However, the reason for the apparent specificity of TRAIL ligand for killing tumor cells remains largely unknown [1].

The physiological role of TRAIL is not well defined, but it has been shown to play a role in T-cell memory, haematopoiesis, autoimmune diseases and many other physiological processes [7-11]. TRAIL plays a major role in the anti-tumor immune surveillance mediated by T-cells and Natural Killer (NK) cells [12, 13]. Indeed, TRAIL was shown to contribute to the regulation of tumor onset, progression and metastasis [14]. TRAIL is mainly expressed at

the membrane level of some immune cells, but can be found in some immune privilege sites [15], or in the circulation in some pathological conditions, including viral infections [16, 17]. Soluble TRAIL, like TNF or sFasL, is less cytotoxic than the membrane-bound form [18]. However, recombinant soluble forms of TRAIL can be used for *in vitro* assays as tagged or non-tagged versions to allow ligand cross-linking and induction of apoptosis.

TRAIL ligand interacts with four distinct receptors at the membrane level, namely DR4 (Death Receptor 4/TRAIL-R1; see ref [19]), DR5 (Death Receptor 5/TRAIL-R2/Killer); see ref [20-23]), DcR1 (Decoy Receptor 1/TRAIL-R3/TRID/LIT [22-25]) and DcR2 (Decoy Receptor 2/TRAIL-R4/TRUNDD; see ref [26, 27]). The two agonistic receptors DR4 and DR5 contain an intracellular Death Domain (DD), which can recruit pro-Caspase-8 and -10 through the adaptor protein FADD (Fas-associated Death Domain) after TRAIL stimulation. The multiprotein complex formed by DR4/5, pro-Caspase-8/-10 and FADD is called the Death-Inducing Signaling Complex (DISC) and leads to the activation of effector caspases for the triggering of apoptosis (Fig. 1).

Depending on the cell type, an amplification loop involving the mitochondria may be required to fully trigger TRAIL-induced cell death [28, 29]. This mitochondrial pathway, also called the intrinsic pathway, is activated after Caspase-8 mediated cleavage of Bid. Truncated Bid activates Bax and Bak, triggering the release of pro-apoptotic factors such as Cytochrome c that lead to the formation of a soluble complex called apoptosome, in which the pro-Caspase-9 is activated. Caspase-9 in turn cleaves and activates Caspase-3, allowing execution of apoptosis. This amplification loop is negatively controlled by anti-apoptotic members of the Bcl-2 family and is heavily dependent on Bax [30], in type II cells [31]. TRAIL-induced apoptosis can also be negatively regulated by other intracellular factors including cellular inhibitor of apoptosis protein (cIAP), X-linked IAP (XIAP) or survivin, downstream of the mitochondrial pathway [32, 33]. Activation of pro-Caspase-8 and -10 within the TRAIL DISC

is tightly controlled by the anti-apoptotic protein c-FLIP (cellular FLICE-like Inhibitory Protein) [34], but its inhibitory function also applies to Fas or TNFR1 [35].

Selective inhibition of the TRAIL pathway can be mediated by the two antagonistic TRAIL receptors, DcR1 and DcR2. Although their exact function is still unclear, these receptors lack a functional DD and are unable to induce the apoptotic program. DcR1 is a GlycosylPhosphatidylinositol (GPI)-anchored protein and does not contain the intracellular DD, whereas DcR2 contains a truncated DD that cannot induce caspases activation [22, 26]. The inhibitory effect of these two antagonistic receptors was initially attributed to their ability to sequester the ligand TRAIL, but it has recently been demonstrated that DcR1 and DcR2 act by two different mechanisms. While DcR1 competes with DR4 and DR5 for TRAIL binding, DcR2 interacts with DR5 within the DISC, and impairs efficient Caspase-8 activation [36]. Another mode of regulation has recently emerged and highlighted the importance of post-translational modifications of death receptors for TRAIL signaling, such as palmitoylation of DR4 [37] or glycosylation of both receptors [38, 39].

Defects in the intrinsic and extrinsic pathways [40, 41], including survival pathways such as NF- κ B or Akt and more generally tumor microenvironment [42], may lead to cell resistance and hamper the future clinical use of TRAIL in oncology. In all of these cases, the threshold of apoptosis induction is too high for efficient cancer therapy, and the current challenge is to decrease this threshold to restore TRAIL functionality.

Thus far, many efforts have been made to find therapeutic strategies that can eradicate cancer cells, without appearance of resistance and without toxic side effects. Some combinatorial strategies have given interesting synergistic activities with TRAIL, for example the use of a broad range of protein inhibitors, chemotherapy or irradiation [43]. More recently, the use of natural compounds, including polyphenols (Table 1), led to a growing interest for these

combined therapeutic approaches, due to their relative safety and their anti-tumor efficacy in preclinical models.

Polyphenols

Polyphenols are the products of secondary metabolism in plants. They play a role in defence mechanisms against pathogens or radiations and give plants their colours. They are found in fruits and vegetables, but also in wine, tea, coffee, chocolate and many other plant-derived products [44]. These compounds are known for their beneficial effects against a large number of diseases, including cardiovascular or neurodegenerative diseases, osteoporosis and cancer [45]. The biological activity of polyphenols is mainly attributed to their antioxidant properties, which is strictly related to their chemical structure [46] (Fig. 2). Polyphenols prevent reactive oxygen species (ROS)-induced DNA damage by scavenging free radicals (reactive oxygen, nitrogen and chlorine species) and by inactivating metal catalysts by chelation, decreasing their oxidative activity. Their ability to interact with other reducing compounds and to inhibit redox-active transcription factors may also contribute to the antioxidant properties of these molecules as well as to their ability to regulate gene expression. Paradoxically, in addition to their antioxidant effects, polyphenols have also been shown to exert pro-oxidant effects that could also be responsible for their anticancer properties [47]. For example, owing to the presence of its hydroxyl groups, the flavonoid quercetin was shown to inhibit proliferation and to induce apoptosis of malignant cells through the generation of intracellular superoxide [48].

Enhancing TRAIL-induced cell death using polyphenolic compounds: Molecular mechanisms

Flavonoids

This group of polyphenols has been identified in fruits, vegetables, grains, roots, flowers, wine, tea and other related products [49]. More than 4000 different flavonoids have been identified, many of which are responsible for the infinite color variations of flowers, leaves and fruits. These compounds are divided into several classes on the basis of their molecular structure, namely anthocyanidins, flavanols, flavonols, flavanones, flavones, isoflavones and chalcones.

Anthocyanidins:

Anthocyanidins and flavanols are the most common flavonoids found in the diet. They are generally found in nature as glycosides, called anthocyanins, and are responsible for the blue, red, or purple colors of plants. Anthocyanin-rich extracts demonstrated chemopreventive activities against cancer in animal models of carcinogenesis [50]. Single agents have been tested in *in vitro* models and have shown interesting anti-proliferative and pro-apoptotic properties, acting by modulating survival pathways such as NFκB [51] or MAPK [52]. So far, their association with TRAIL has not been documented, albeit the pro-apoptotic activity of prodelphinidin B-2 3,3'-di-O-gallate, a proanthocyanin, was proposed to proceed through the concurrent upregulation of Fas and FasL [53]. It remains to be demonstrated whether this group of flavonoids might prove useful to sensitize resistant cancer cells to TRAIL. Since anthocyanins are more stable than their aglycone anthocyanidins [54], the former should be preferred to assess their suitability in preclinical settings before considering these compounds for future clinical use.

Flavanols:

The richest sources of flavanols are green tea, chocolate, red wine and many types of fruits. They are found as monomers, called catechins, or as polymers, commonly called condensed tannins. The only compound tested in association with TRAIL is Epigallocatechin-3-gallate (EGCG) (Fig. 2A). Nishikawa *et al.* were the first to show the synergistic effect of the

association of EGCG with TRAIL in human hepatocellular carcinomas [55]. The authors attributed this effect to the inhibition of Bcl-2 and Bcl-XL by EGCG, probably through NFκB inhibition. In this study, no apparent change in the survivin, XIAP, c-IAP1 or c-FLIP expression levels were observed. Of note, the amount of EGCG used in this work was high (100 μg/mL) and the safety of such a concentration on normal cells was not addressed. Later, two different teams demonstrated the efficiency of EGCG, used at lower concentrations, on glioblastomas [56] or prostate carcinomas [57]. Siegelin *et al.* observed that sensitization of glioblastomas to TRAIL-induced apoptosis occurred through EGCG-mediated Akt inhibition, leading to the downregulation of survivin and the Death Effector-Domain (DED)-containing protein PEA15 [56]. DR4 and DR5 were not regulated by EGCG at the protein level, but since their expression at the membrane level was not assessed, it cannot be excluded that these receptors might partially contribute to the synergy. On the contrary, another study by Siddiqui *et al.* demonstrated that similar concentrations of EGCG induced an increase in DR4 expression on prostate carcinomas [57]. Many other proteins were modulated in this model, such as Bcl-2, Bcl-XL, IAP proteins, survivin, c-FLIP, Bad, Bax, Bak and Smac/Diablo, but their relative requirement regarding cell sensitization to TRAIL was not assessed. Recently, another paper revealed that EGCG upregulated DR4 and DR5 and promoted TRAIL-induced cell death of pancreatic carcinomas [58]. The human melanoma A375 cell line was also shown to be responsive to the combinatorial treatment, but the molecular mechanisms were not clarified [59].

Flavonols:

This flavonoid subgroup is abundant in a variety of foods including onion, broccoli, apple, curly kale, leek and tea. To date, flavonols that have been described to exhibit TRAIL-sensitizing properties are quercetin, kaempferol and myricetin (Fig. 2A).

Quercetin has been widely studied for its anti-oxidative action and its effect on the expression of many genes. It has been reported to confer protection against disorders such as neurodegeneration, cardiovascular diseases or cancers [60]. In 1996, a phase I clinical trial showed that quercetin can be safely administered by intravenous injection [61]. Several studies have been performed investigating the effects of associating TRAIL with quercetin. Kim *et al.* showed that this co-treatment efficiently killed prostate carcinomas but not normal prostate cells [62], though the concentrations of quercetin used in this study were very high. Quercetin's sensitizing activity was associated with its ability to inhibit the Akt pathway. The authors subsequently demonstrated that quercetin enhanced TRAIL-induced apoptosis by downregulating survivin through a mechanism involving ERK-MSK1-mediated deacetylation of histone H3, independently of the MAPK and the JNK pathways [63]. They also showed that quercetin did not change the protein levels of the TRAIL receptors, c-FLIP, IAP proteins, Bcl-2, Bcl-XL and Bax. Russo *et al.* reported that leukemia cell lines were efficiently sensitized by quercetin and TRAIL co-treatment, except for the T-cell acute lymphoblastic leukemia HPB-ALL [64]. At the same time, Chen *et al.* elegantly demonstrated that pre-treatment with quercetin sensitized non-small cell lung cancer cells to TRAIL-induced apoptosis *via* two distinct mechanisms [65]. On one hand, quercetin acted on the Akt pathway to downregulate survivin expression and, on the other hand, quercetin targeted the PKC kinase, leading to an increase in DR5 expression. In their settings, neither c-FLIP nor IAP family members were modulated and interestingly, normal bronchial epithelial cells were not affected by this treatment. Another report showed that quercetin stimulated DR5 expression and synergised with TRAIL to kill six different hepatoma cell lines [66]. Quercetin-mediated DR5 upregulation was triggered by Sp1. Supporting Chen's results, this transcription factor was shown to be itself under the control of PKC [67]. Moreover, this study is probably the only instance in the literature to report a diminution of the short isoform of c-FLIP by

quercetin, through proteasomal degradation. The synergistic activation of the TRAIL pathway by quercetin has also been associated with the cell surface redistribution of TRAIL receptors. Indeed, while Psahoulia *et al.* observed no particular change in DR4 and DR5 expression levels on colon adenocarcinomas after quercetin pre-treatment, they found out that these receptors were redistributed into lipid rafts, thus facilitating TRAIL DISC formation and initiator caspases processing [68]. However, as far as TRAIL receptor partitioning to lipid rafts is concerned, conclusions regarding enhanced DISC formation in these particular membrane compartments should be moderated as DISC analysis was performed using a detergent that is unable to solubilise lipid rafts. Alternatively, mitochondrial activation or post mitochondrial events were also shown to contribute to the synergy. Siegelin *et al.* supported the importance of survivin in quercetin-induced sensitization to TRAIL on various glioma cell lines [69]. In four glioma cell lines that displayed good synergistic apoptotic activities upon TRAIL and quercetin treatment, the authors observed a proteasomal degradation of survivin mediated by inhibition of Akt, whereas in gliomas resistant to the combined treatment, survivin levels remained unchanged. In this model, the ability of quercetin to regulate survivin expression appeared to be the major event governing the efficiency of the combined treatment. Finally, Hasegawa *et al.* assessed the efficiency of methylidihydroquercetin, a methylated version of quercetin called BB-1, extracted from the Asian medicinal plant *Blumea balsamifera* [70]. Similar to quercetin, BB-1 enhanced TRAIL-induced apoptosis in six different leukemia cell lines. Upon methylidihydroquercetin pre-treatment, DR5 was upregulated at the membrane level, but contrary to the observation of Chen *et al.*, PKC was not required for this regulation. In addition, while a downregulation of active Akt and a modulation of transcription factors such as c-Rel and p52 were observed, the levels of survivin, Bax, Bak, Bcl-2, Bcl-XL, XIAP, c-IAP1, c-IAP2 and c-FLIPs remained unaffected

in this study. Interestingly, this treatment was not toxic against normal peripheral blood mononuclear cells (PBMC).

Kaempferol is another flavonol that has been described to facilitate TRAIL-induced apoptosis. Resistant colon carcinomas were efficiently eradicated by this co-treatment through a mechanism involving DR4 and DR5 upregulation, but in the absence of XIAP, survivin, Bcl-XL or Bax regulation [71]. Interestingly, while silencing of DR5 blocked the synergy, silencing of DR4 was unable to protect tumor cells to the combination. However, it should be noted that the membrane levels of DR5 or DR4 was not assessed in this study, so the absence of blockade after DR4 silencing could be explained by a lack of DR4 at the cell surface. Consistent with a previous study [72], Bcl-2 was surprisingly upregulated by kaempferol without compromising TRAIL-induced cell death. Association of kaempferol with TRAIL was also effective in the prostate carcinoma cell line PC-3 [71], and in three out of seven gliomas [73]. The sensitization was associated in gliomas with a decrease in XIAP, Bcl-2 (but not Bcl-XL), Mcl-1 and with the proteasomal-mediated degradation of survivin, due to Akt inhibition. Kaempferol and TRAIL co-treatments were unable to engage the apoptotic machinery in normal hepatocytes [71].

Another flavonol of interest for combination with TRAIL therapy is myricetin. It has been revealed to exert synergistic pro-apoptotic effects when combined with TRAIL at subtoxic doses on glioma cells, but not on normal astrocytes [74]. During synergy, both c-FLIP_S and c-FLIP_L were downregulated, as well as Bcl-2. Interestingly, overexpression of Bcl-2 or c-FLIP_S (but not c-FLIP_L) attenuated TRAIL-induced cell death in cells co-treated with myricetin, indicating that these inhibitory proteins are relevant for the control of the synergy. Here, sensitization to TRAIL was independent of the tumor suppressor P53 because P53-mutant gliomas were as responsive as wild type P53 expressing gliomas.

Flavanones:

Hesperetin, naringenin and eriodictyol are the most abundant flavanones found in plants, mainly in citrus fruits. The validity of this kind of flavonoid in combination with TRAIL remains to be assessed.

Flavones:

The flavones subgroup of flavonoids is not widely distributed in plants but is abundant in celery, parsley and some herbs. Apigenin, luteolin, wogonin and baicalein were studied in association with TRAIL and were shown to exhibit interesting combinatorial properties (Fig. 2A).

Apigenin was the first flavone revealed to sensitize breast cancer cells to TRAIL-induced apoptosis [75]. This phytochemical was shown to inhibit Casein kinase II, leading to inhibition of NF κ B-mediated expression of Bcl-XL and c-FLIP. The synergy appeared to be largely dependant on Bax but independent of *p53*, as demonstrated on colon carcinomas deficient either for Bax or for *p53*. Later, Horinaka *et al.* reported the suitability of this co-treatment on lymphoblastic leukemia (Jurkat), metastatic prostate carcinoma (DU145) and colon carcinoma (DLD-1) [76]. They described a new mechanism of action of apigenin, based on DR5 stabilization independent of *p53*. The co-treatment was safe toward normal PBMC *in vitro*, and DR5 was not modulated by apigenin on these non-cancerous cells.

Horinaka *et al.* also studied the flavone luteolin, in human cervical cancer HeLa cells [77]. Cleavage of Bid was observed during co-treatment with TRAIL and luteolin, as well as an increase in the expression of the TRAIL receptor DR5. While the synergy was blocked by DR5 silencing, underlying the importance of this receptor, the implication of DR4 was not analyzed in this study. However, the authors demonstrated that the combination of luteolin and TRAIL is safe for normal PBMC. Concurrently, Shi *et al.* demonstrated another mechanism of sensitization, independent of any regulation of the TRAIL receptors or Bcl-2, Bcl-XL, c-FLIP, c-IAP1, c-IAP2 or NF κ B [78]. In their study, cervical, colon, liver or

nasopharyngeal carcinomas were greatly sensitized to TRAIL-induced cell death upon a short exposure to luteolin. Sensitization was attributed to the inhibition of PKC, which led to increased XIAP ubiquitination and degradation. Thus, the authors proposed that luteolin could be a potent compound to overcome TRAIL resistance in cancers that exhibit elevated PKC activity.

Wogonin is another flavone shown to sensitize different lymphomas to TRAIL or TNF α -induced cell death, without having effects on normal peripheral blood T cells [79]. Wogonin was shown to inhibit TNF α -induced NF κ B activation in a reactive oxygen species (ROS)-independent manner, but it was not confirmed for TRAIL. More recently, Lee *et al.* underlined the importance of P53, Puma and Bax during co-treatment with TRAIL and wogonin, using prostate and colon carcinomas deficient for each of these proteins [80]. A schematic model of action was proposed, where wogonin induced ROS production and subsequent P53 activation. The histone H2A.X was also phosphorylated, which is a typical feature of DNA damage. These events led to the upregulation of Puma and the activation of the mitochondrial pathway [81]. In another study, wogonin was shown to be unable to sensitize four different pancreatic carcinomas to the combined treatment with TRAIL [82], but these cells are known to bear *p53* mutations. This fact could explain, at least in part, the relative cell specificity of the combined treatment. Finally, wogonin has been shown to be safe when administered intravenously to dogs [83], and could be an interesting therapeutic candidate in association with TRAIL for tumors harbouring a functional P53 protein

Baicalein is a flavone originally isolated from the roots of the Asian medicinal herb *Scutellaria baicalensis*. This molecule was studied for its anticancer potential when associated with TRAIL on prostate, colon, T-cell leukemia and hepatoma cancer cell lines [84], and the combination was not toxic against normal blood cells and hepatocytes. Baicalein-induced sensitization to TRAIL was shown to be mediated *via* two different mechanisms, namely

ROS induction or upregulation of CHOP transcription factor, depending on the cell type. Both pathways led to an increased expression of DR5 that could explain the synergistic effect.

Isoflavones:

Isoflavones are commonly referred as phytoestrogens because of their structural similarities with estrogens. Particularly, these molecules can bind estrogen receptors and mimic their effects [85]. Leguminous plants are almost the exclusive source of isoflavones, such as genistein or daidzein (Fig. 2A).

The ability of genistein to enhance TRAIL-induced cell death was first demonstrated on lung carcinomas, through downregulation of both Akt and Bcl-XL [86]. An *in vivo* study in mice transplanted with human pancreatic cancer cells revealed that genistein co-treatment with TRAIL triggered a significant reduction of tumor volume, without toxic side effects [87]. The effectiveness of genistein was explained in cervical cancer Hela cells by its ability to inhibit the ERK pathway [88]. Genistein was reported to enhance TRAIL killing of hepatocellular and lung carcinomas, *via* inactivation of the p38 MAPK signaling and activation of the mitochondrial pathway [89, 90]. Gastric adenocarcinomas were also greatly sensitized, as demonstrated by Jin *et al.*, in a way involving mitochondrial activation, DR5 upregulation and Bcl-XL downregulation, without affecting the levels of Bcl-2 and IAP proteins [91]. On the contrary, Siegelin *et al.* showed that genistein decreased the levels of Bcl-2, XIAP, survivin and active Akt, and enhanced the proteasomal degradation of c-FLIP_s, in glioblastoma cells [92]. Interestingly, normal human astrocytes were not sensitized to TRAIL-induced cell death by genistein. While these findings are interesting *in vitro* for the treatment of glioblastomas, it remains to be determined whether genistein can cross the blood-brain barrier.

Only one study has reported the synergistic effect of the isoflavone daidzein in association with TRAIL against glioblastoma cancer cells [93]. Siegelin *et al.* demonstrated that this

synergy was essentially mediated *via* Bcl-2 downregulation, whereas c-FLIP, XIAP, survivin or the TRAIL receptors were not modulated.

Chalcones

Chalcone is the first compound in flavonoid biosynthesis and thus is widely found in all kind of plants. Two chalcone derivatives have been tested in combination with TRAIL, namely butein and isoliquiritigenin (Fig. 2A).

Butein, a chalcone purified from the barks of the lacquer tree *Rhus verniciflua*, was reported to enhance TRAIL-promoting effects on monoblastoma and leukemia cell lines, through an increase in DR5 expression at the membrane level [94]. This compound could be interesting for future clinical protocols because no toxicity was observed in normal lymphocytes and CD34+ cells from healthy donors.

Isoliquiritigenin is a chalcone derivative found abundantly in licorice. Co-treatment with subtoxic doses of isoliquiritigenin and TRAIL efficiently killed colorectal HT29 cells [95]. Expression of Bcl-2 and Bcl-XL was not changed, but a slight increase of DR5 was reported. However the importance of DR5 during the synergy was not assessed and the mechanism of action need to be further explored.

Stilbenes

Stilbenes are produced by plants that are subjected to various stressful conditions. Resveratrol has been widely studied for several years due to its anti-carcinogenic properties (Fig. 2B). Since 2003, ten different studies have assessed the potential of resveratrol to sensitize resistant cancer cells to TRAIL-induced apoptosis (Table 1). Resveratrol, at a concentration of 10 μ M, was initially shown to be unable to sensitize prostate LNCaP cells to TRAIL [96]. In two other studies using ten fold higher concentrations, Fulda and Debatin reported that resveratrol sensitized various cancer cells to TRAIL-induced apoptosis, including

neuroblastoma, medulloblastoma, glioblastoma, melanoma, T-cell leukemia and pancreatic, breast and colon carcinomas [97, 98]. Sensitization was associated with a p21-mediated cell cycle arrest and concomitant survivin depletion, independent of P53. Untransformed human fibroblasts remained insensitive to the co-treatment. Delmas *et al.* demonstrated that, while their expression levels remained the same, partitioning of the TRAIL receptors into lipid rafts was responsible for resveratrol's sensitizing activity in two carcinoma cell lines [99]. Later, Shankar *et al.* reported that resveratrol sensitized prostate carcinomas to TRAIL by way of the modulation of many molecular targets [100, 101]. The expression of survivin, Bcl-2, Bcl-XL and XIAP was inhibited while the expression of DR4, DR5, Bim, Bax, Bak, Noxa and PUMA was increased. Of note, sequential treatments were more efficient than co-treatment in triggering cell death in this study. Importantly, the combination was not toxic against normal prostate epithelial cells. Contrary to these results, Gill *et al.* observed no major changes regarding DR4, DR5, Bcl-2, Bcl-XL, survivin and XIAP on the same prostate cancer cell lines [102]. In their hands, c-IAP1 was downregulated but seemed to be only partially involved, because cIAP-1 siRNA alone did not restore the full sensitivity to TRAIL. The synergy could also be explained by the increased expression of Bax that could change the Bax/Bcl-2/Bcl-XL ratio, or upstream by the observed inactivation of the Akt pathway. Using the same prostate cells, Sallman *et al.* proposed that the combined effect of resveratrol and TRAIL is mediated by the inhibition of the src/jak-Stat1 pathway [103]. According to their study, resveratrol decreased Clusterin expression by inhibiting the phosphorylation of the Src and Jak kinases, resulting in loss of Stat1 activation. Using different melanoma cell lines, Ivanov *et al.* also demonstrated the efficacy of resveratrol associated with TRAIL [104]. They observed that the JNK-cJun and MAPK p38-ATF2 pathways were activated upon resveratrol pre-treatment, as well as many downstream targets such as c-FLIP, Bcl-XL, survivin and Cyclin D1. Moreover, normal human lung fibroblasts and melanocytes remained protected

from apoptosis. Despite many promising investigations regarding the effect of resveratrol, the TRAIL-enhancing properties of this phytochemical might depend on the cellular context. For example, researchers shown that three pancreatic carcinomas were resistant to the combined treatment, whereas one other cell line exhibited only a slight additive effect [82].

Lignans and flavonolignans

Plant lignans are polyphenolic compounds which are referred as phytoestrogens, like isoflavones. Flavonolignans, such as silibinin, are heterodimers formed through the coupling of a flavonoid and a lignan component (Fig. 2C).

Nordihydroguaiaretic acid (NDGA) is a phenolic lignan that has been considered as a lipoxygenase inhibitor. This molecule was shown to enhance the pro-apoptotic effect of TRAIL in leukemia Jurkat cells, SW480 colon carcinomas and prostate cancer DU145 cells, without affecting normal PBMC [105]. The increased expression of DR5 at the membrane level was proposed to explain the synergy because most of the major apoptosis-related proteins such as Bcl-2, Bcl-XL, survivin, c-IAP1 and XIAP remained unaffected.

Honokiol is a lignan originally isolated from *Magnolia officinalis*. Raja *et al.* reported an interesting synergy with TRAIL on non-small cell lung cancer cell lines, which was accompanied by the modulation of proteins such as DR4, DR5, survivin and c-FLIP [106]. However, some clues highlighted the prevalence of c-FLIP regulation during honokiol and TRAIL co-treatment. Actually, c-FLIP inhibition appeared to be the earlier event, and its overexpression strongly abolished the synergy. The researchers went a step further in the experiment by testing different honokiol derivatives. They observed a correlation between the efficacy of these molecules and their ability to inhibit c-FLIP. Therefore, c-FLIP appeared to be critical for honokiol-mediated sensitization to TRAIL in this model.

Silibinin from the herb milk thistle was tested with TRAIL on glioma cells [107]. This flavonolignan exerted significant TRAIL-enhancing effects on gliomas but not on normal astrocytes. During synergy, DR5 appeared to be upregulated *via* the transcription factor CHOP, whereas survivin and c-FLIP were downregulated.

Phenolic acids and derivatives

Plant phenolic acids are derived either from benzoic acid or cinnamic acid (Fig. 2D). Curcumin is a hydroxycinnamic acid derivative responsible for the yellow color of the spice turmeric. Anticancer action of curcumin has given rise to many publications that have revealed a broad spectrum of molecular targets [108, 109]. Co-treatment with TRAIL has also been well studied since 2003 (Table 1). Deeb *et al.* demonstrated in human prostate carcinomas that curcumin-mediated sensitization to TRAIL involved both the activation of mitochondria [96] and the inhibition of NF κ B signaling [110]. Curcumin was shown to block I κ B α phosphorylation, thus allowing sustained sequestration of NF κ B into the cytosol [111]. The combination of curcumin with TRAIL appeared to be safe for non-tumorigenic prostate epithelial cells. The authors further pointed out that in prostate cancer cells with high level of active Akt (LNCap and PC-3), sensitization to TRAIL by curcumin was mediated by the suppression of NF κ B *via* inactivation of the Akt pathway. In prostate cancer cells that lack basal active Akt (DU145), sensitization was also mediated by inhibition of NF κ B through the suppression of I κ B α phosphorylation, but independently of Akt [112]. In both cases, NF κ B inhibition by curcumin resulted in the inhibition of Bcl-2, Bcl-XL and XIAP expression. These results were confirmed *in vivo*, as prostate PC3 cells xenografted in mice were efficiently killed upon co-treatment [113]. Shankar *et al.* also studied the effect of curcumin and TRAIL on prostate carcinomas. They confirmed the observations of Deeb *et al.* concerning the downregulation of XIAP, Bcl-2 and Bcl-XL, but showed in addition that

survivin and Noxa were also inhibited whereas the levels of Bax, Bak, Bim, PUMA, DR4 and DR5 were augmented [114]. *In vivo* experiments performed on nude mice xenografted with prostate LNCAP cells revealed that treatment with a combination of curcumin and TRAIL inhibited tumor growth by apoptosis triggering, but also through the activation of anti-proliferative, anti-angiogenic and anti-metastatic mechanisms [115]. Many proteins were positively regulated, such as DR4, DR5, Bax, Bak, p21 and p27, whereas NF κ B was negatively regulated as well as its various gene products, including Bcl-2, Bcl-XL, and Cyclin D1. In contrast, Jung *et al.* demonstrated that in renal and hepatocellular carcinomas, curcumin and TRAIL mediated apoptosis occurred with no change in expression of Bcl-2, Bax, c-IAP2 or XIAP [116]. The synergy was proposed to be induced *via* a ROS-mediated DR5 upregulation, and the combination was safe toward non-malignant mesangial cells [117]. However in these settings, the transcription factor CHOP, although activated by ROS, was neither associated with curcumin-mediated DR5 upregulation nor with the synergistic activity of the combination. The mitochondrial pathway was proposed to play a minor role because Bcl-2 overexpression in hepatocarcinomas failed to interfere with the sensitization. Using Burkitt's lymphoma cells lacking a functional Bax protein, Hussain *et al.* came to similar conclusions [118]. However, the mitochondrial pathway was still activated upon co-treatment in Bax-deficient lymphomas, suggesting that Bak may compensate for the loss of Bax. Sensitization to TRAIL in these cells was also associated with the upregulation of DR5 and the inactivation of NF κ B in a ROS dependent manner. The efficacy of curcumin and TRAIL association was also demonstrated in gliomas [119], bladder [120] and ovarian carcinomas [121]. Thereby, curcumin could be a potent phytochemical in order to overcome TRAIL-resistance in diverse cancer types.

Recently, another phenolic compound called cycloartenyl ferulate, extracted from rice bran oil, has been shown to potentiate TRAIL-induced cell death, which is associated with the upregulation of DR4, DR5 and Bak and the decrease in Bcl-2 expression [122].

Synthetic flavonoids

Given that naturally occurring flavonoids are interesting compounds for anticancer therapy or chemoprevention, researchers aimed to design synthetic versions that can be produced in large amounts. Thus, a semi-synthetic flavone called flavopiridol has been developed by Aventis (Fig. 3), and has been shown to display both *in vitro* and *in vivo* selective antitumor activity [123]. Phase I and II trials have demonstrated the absence of cytotoxicity of this molecule [124]. Flavopiridol was shown to efficiently synergize with TRAIL in several cancer types including cholangiocarcinomas [125] and leukemia cell lines [126] through the respective downregulation of Mcl-1 or XIAP. In hepatocarcinomas, co-administration of flavopiridol and TRAIL resulted in a decline of survivin expression and an increase of DR4 and DR5 at the membrane level [127]. In contrast, breast cancer cells were mainly sensitized by an enhanced formation of the TRAIL DISC and a downregulation of c-FLIP [128]. Likewise, a deregulation of c-FLIP was observed in myeloma and breast cancer cells [129]. Flavopiridol antitumor activity with TRAIL has also been reported in lung carcinomas [130].

Concluding remarks

The interest for natural substances in the treatment of cancer is not recent [131]. Many existing drugs have been discovered from plants used in traditional medicine. For the moment polyphenols are not yet used in anticancer therapy, but they are currently used as dietary supplements because of their known preventive properties against ageing, neurodegeneration, cardiovascular risk or cancer. The anticancer efficacy of polyphenols in association with

chemotherapy, radiotherapy [132], or in combination with TRAIL [133, 134] in preclinical models is now well demonstrated. The molecular events driving tumor cell sensitization to TRAIL-induced cell death generally target both the intrinsic and the extrinsic pathways. In most cases, polyphenols are able to lower the threshold of caspase activation, thus allowing TRAIL-induced apoptosis triggering. Considering all the data published so far, it is difficult to define a general rule concerning the molecular action of each polyphenol. Rather, it appears that molecular events that allow engagement of the apoptotic machinery upon combined treatments largely depend on the cancer type, and on the signaling pathways engaged in controlling cell resistance to apoptosis. For example, in cancer cells that are resistant to TRAIL through a blockage of the intrinsic pathway, polyphenolic compounds are generally able to inhibit anti-apoptotic proteins of the Bcl-2 family such as Bcl-XL, Bcl-2 or Mcl-1, or to upregulate pro-apoptotic members such as Bax or Bak (Fig. 4). Likewise, inhibition of the Akt pathway by polyphenols can lead to the restoration of the mitochondrial pathway, affording an enhanced sensitivity of the tumor cells to TRAIL-induced cell death (Fig. 4). On the other hand, when the resistance occurs at the level of the extrinsic pathway, the synergistic activity of these polyphenols is often associated with the upregulation of the agonistic TRAIL receptors, the downregulation of c-FLIP, or through a facilitation of TRAIL receptor aggregation and DISC formation (Fig. 4). Interestingly, most of these synergistic activities appear to occur independently of *p53*, a tumor suppressor gene that confers resistance to anticancer treatments if mutated or deleted. Aside from polyphenols, it is relevant to mention that other agents with related structures, although not polyphenolic, could also sensitize cells to TRAIL by similar mechanisms of action, such as the triterpenoids celastrol [135, 136] and lupeol [137].

Overall, the finding that polyphenols exert multimodal sensitizing activities is very encouraging for future combined treatments with TRAIL or TRAIL derivatives. However it is

crucial to be cautious about the clinical relevance of these *in vitro* studies. Most *in vitro* studies are likely to be relevant for intravenous administration protocols in future clinical uses, but not for nutritional intake of a polyphenol-rich food. Indeed, *in vitro* studies are often performed with high concentrations of polyphenol that in no way mimic the concentration at the tumor site following oral delivery [138]. Moreover, the compounds tested in these studies are almost always native polyphenols, but these non-conjugated forms are found at low levels after oral administration due to fact that polyphenols are rapidly absorbed and metabolised, mainly as sulfates, glucuronates or methylated forms [139]. Thus, almost all the data collected correspond to a direct injection of polyphenols into the blood, but not to a dietary supplementation. The poor stability of polyphenolic compounds within the blood, although problematic, could nonetheless be resolved using various galenic formulations, such as calcium-pectinate beads or liposome-based delivery strategies [140, 141]. For example, intratumor injection of EGCG after encapsulation into liposomes provided interesting results in a mouse model of human cancer [142]. Liposomes protected EGCG from degradation and increased cell death in melanomas and colon carcinomas compared to free EGCG. Thus, liposomes could allow the use of lower doses of polyphenol and possibly reduce side effects without reducing their anticancer efficacy. Accordingly, it has been demonstrated that encapsulation of quercetin into liposomes drastically increases its lifetime in plasma, and allows increased quercetin delivery to the tumor, leading to cancer growth inhibition in tumor-bearing mice after intravenous injection [143]. Interestingly, liposome-mediated quercetin delivery into the kidney and the lungs was lower as compared to free quercetin, a finding that may prove useful to limit treatment side effects. According to the authors, the tumor-selectivity of these liposomes could be explained by the presence of defects in the capillary endothelium of tumoral tissues, which allow their extravasion from the blood to the

tumor cells [143]. Therefore, the use of liposomes for intravenous administration of polyphenols could be relevant for the treatment of solid tumors.

Alternatively, direct administration by application onto the skin may be considered in the case of skin cancers, including melanomas. Indeed, topically applied resveratrol was shown to be a safe and efficient approach for transdermal delivery of active polyphenols into the skin [144].

These preliminary *in vitro* studies are very promising and encouraging for the use of polyphenols and TRAIL for future therapeutic protocols. Both compounds exert specific antitumor activity, and the combination of the two molecules is proven safe for normal cells, at least *in vitro*. Thus, future work *in vivo* should help our understanding of the different mechanisms for synergy, and ultimately clinical trials will reveal whether a combination treatment utilising TRAIL and natural polyphenols represents a realistic and effective anticancer therapy.

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Table 1 Classification of polyphenols that have been shown to exhibit a synergistic effect with TRAIL.

Classification		Compounds	References
Flavonoids	Anthocyanidins	<i>ND</i>	
	Flavanols	Epigallocatechin gallate	[55-59]
	Flavonols	Quercetin	[62-66, 68, 69]
		Methyldihydroquercetin BB-1	[70]
		Kaempferol	[71, 73]
		Myricetin	[74]
	Flavanones	<i>ND</i>	
	Flavones	Apigenin	[75, 76]
		Luteolin	[77, 78]
		Wogonin	[79, 80, 82]
		Baicalein	[84]
		Flavopyridol (semi-synthetic flavone)	[125-130]
	Isoflavones	Genistein	[86-92]
Daidzein		[93]	
Chalcones	Butein	[94]	
	Isoliquiritigenin	[95]	
Stilbenes	Resveratrol	[82, 96-104]	
Lignans / Flavonolignans	Nordihydroguaiaretic acid	[105]	
	Honokiol	[106]	
	Silibinin	[107]	
Phenolic acids and derivatives	Curcumin	[96, 110-121]	
	Cycloartenyl ferulate	[122]	