

Vanilloid-mediated apoptosis in prostate cancer cells through a TRPV-1 dependent and a TRPV-1-independent mechanism

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Abstract. Vanilloids are natural molecules identified in a plethora of foods normally ingested through the diet. They mediate apoptosis through a direct pathway (independent of TRPV-1, the receptor for vanilloids) and through an indirect pathway, i.e. thanks to the interaction with TRPV-1 and the successive intracellular calcium growth $[Ca^{2+}]_i$. Some vanilloids, such as capsaicin, dihydrocapsaicin and resiniferatoxin (the ultrapotent analogue of capsaicin, extractable from *Euphorbia resinifera*), may be considered as coenzyme Q antagonists: in fact, they inhibit the run of the electrons through the electron transport chain, so determining an excess of reactive oxygen species (ROS). A second effect of the interaction between the vanilloids and TRPV-1 receptor may be reported: it is the fast decrease of the transmembrane mitochondrial potential ($\Delta\Psi_m$). Through the direct pathway, on the contrary, the vanilloids induce apoptosis also interacting with caspases, particularly caspase 1 and 3. On the whole, the vanilloids are able to lead to the intracellular calcium growth and consequently to the evidence of precocious and late elements of apoptosis. (www.actabiomedica.it)

Key words: Vanilloid, capsaicin, prostate cancer, apoptosis, TRPV-1

Introduction

Vanilloids are natural molecules identified in a plethora of foods that are normally ingested through the diet. In particular, they are found in spicy foods, such as hot chili pepper, and belong to the family of *Solanaceae*, genus *Capsicum*, in which the molecule responsible for the spicy taste is capsaicin (*trans*-8-metil-*N*-vanillyl-6-nonenamide), which was identified by Monsereenusorn in 1982 (1).

Some other vanilloids are found in other vegetables belonging to the same family. The ultrapotent analogue of capsaicin is noteworthy: it is known as resiniferatoxin and derives from *Euphorbia resinifera*.

Especially in the last decade, the role of capsaicin in tumoral growth and cell transformation represents a much-discussed question in scientific literature. Ini-

tially, there were evidences that made scientists suppose that vanilloids had an anti-apoptotic role; successively, it was realized that these molecules not only have a role against tumoral growth and transformation, but they also make apoptosis possible in cells in which the process of tumoral transformation has already begun.

In our literature review, we are especially interested in androgen-dependent prostate cancer cells (LNCaP) and androgen-independent prostate cancer cells (PC-3). In these cells, many experiments described in literature demonstrate that capsaicin and other vanilloids induce apoptosis through two different pathways: directly, i.e. through a receptor-independent mechanism, and indirectly, i.e. by a TRPV-1 (Transient Receptor Potential Vanilloid – 1) receptor-mediated mechanism.

Biochemical structure of the vanilloid receptor

More than twenty different genes encode for six groups of receptorial molecules: TRPC, TRPP, TRPV, TRPM, TRPN and mucolipins. All of these are receptors capable of producing a transient potential in the plasmatic and mitochondrial membrane (2-4). TRPV is the vanilloid receptor: it was identified thanks to the study of Caterina and Julius (5) on pain modulation. In the biochemical structure of the receptor, the subunit 1, also called TRPV-1, an ionotropic channel with regulatory functions, is very important (6).

This receptor is mainly expressed in the spinal cord and in trigeminal ganglia (7), but also in urothelium (8), in smooth muscle and in cheratinocytes: it is generally identified on the plasmatic membrane of nociceptive nervous cells in correlation with unmyelinated C fibers or, less frequently, with A δ fibers. In addition, TRPV-1 also plays a role in neurogenic inflammation and in visceral pain, such as in bladder tissue, in which it attenuates hyperalgesia and visceral pain responses (9).

TRPV-1 is made up of six trans-membrane segments with a pore region between the fifth and the sixth segment: four N-termini extremities interact with the citosol (2). The central pore is made up of an intracellular region composed of the α -helices of the sixth domain (D6) and an extracellular region composed of four successive loops (Fig. 1).

TRPV-1 is a non-selective cationic channel activated by vanilloids, in which Asp646 and Glu636, two different aminoacidic residues faced on the internal tunnel of the pore are key molecular determinants for the in-coming and out-coming of ions, water and other ligands (3).

Chemically, vanilloids are lipophilic molecules and are known to have three functional regions: an aromatic region, a region linking ester or amide, and an aliphatic region. The linking with the TRPV-1 receptor is possible thanks to the aliphatic region. Nevertheless, vanilloids can interact with TRPV-1 through the intracellular face of the receptor, thanks to domain 2 and domain 3: in these domains an aminoacidic residue of tyrosine in position 511 seems to play a key role in capsaicin linking (10).

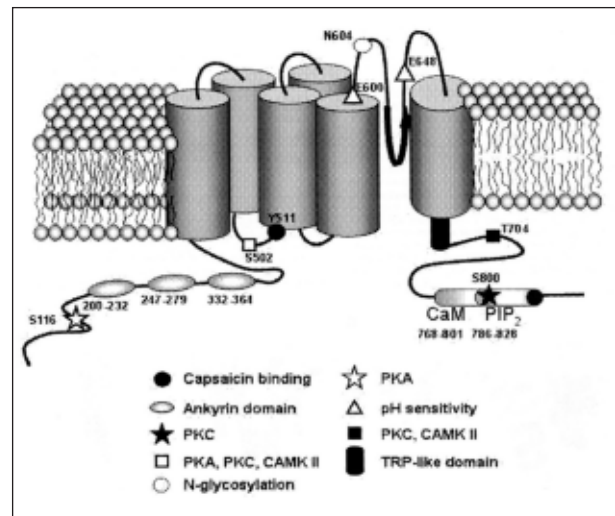


Figure 1. Biochemical structure of TRPV-1

The intracellular domain of TRPV-1 contains a binding site for phosphatidylinositol-4,5-bisphosphate (PIP₂) and two other sites for a protein-kinase C and a CAM-kinase II: the binding site for PIP₂ is important in the channel regulation, as demonstrated by position of the phosphorylation site of protein-kinase C, exactly in the middle of the PIP₂ binding site (3).

Functional properties of vanilloids

Vanilloids are natural molecules identified in a plethora of foods that are normally ingested through the diet (Figs. 2-4). Biologically, they have been identified in the plasmatic membrane double layer, and contribute to determine the structure and fluidity. They are very lipophilic molecules, which besides determining membrane characteristics, contribute to redox homeostasis of the cell and play a role in cell interaction with proteins and lipids on the extracellular or intracellular layers (11).

The function of vanilloids in cellular activity is at two different levels: through the above mentioned interaction with the TRPV-1 receptor, or through a direct interaction with the cell thanks to their ability to across the plasmatic membrane bilayer due to their lipophily.

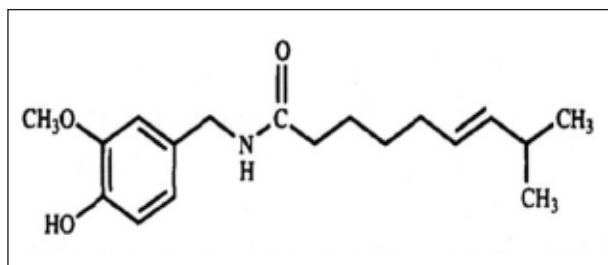


Figure 2. The chemical structure of capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide)

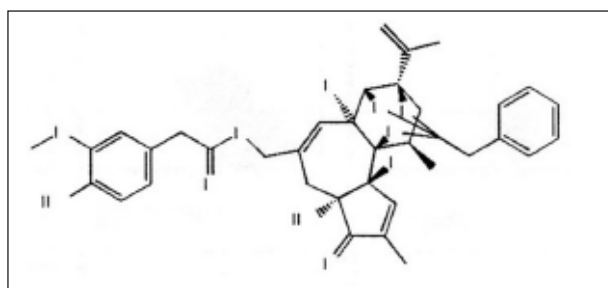


Figure 3. Resiniferatoxin

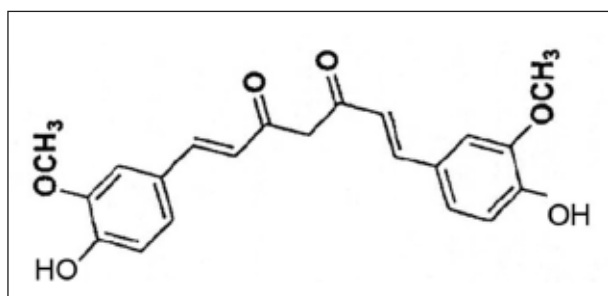


Figure 4. Curcumin

TRPV-1 is mainly identified in the nervous system: in this tissue, the interaction with vanilloids is a key point in pain responses, especially the nociceptive ones (6). This is the reason why the interaction capsaicin/TRPV-1 may be pharmacologically useful in order to relieve neurogenic and inflammatory pain. On the contrary, in other cells, e.g. prostatic cancer cells, the interaction with the TRPV-1 receptor determines a series of intracellular events leading to apoptosis. Moreover, the TRPV-1 independent interaction also has a specific role in inducing apoptosis. Cap-

saicin, such as other vanilloids, induces apoptosis in tumoral cells through a TRPV-1 independent mechanism, thanks to the growth of reactive oxygen species (ROS) and consequently the alteration of mitochondrial transmembrane potential ($\Delta\Psi_m$) (12).

Direct pathway leading to vanilloid-mediated apoptosis

Vanilloids mediate apoptosis through a direct pathway (independent of TRPV-1) and through an indirect pathway (13), i.e. thanks to the interaction with TRPV-1 and the successive intracellular calcium growth [Ca^{2+}].

Regarding the direct pathway, it may be demonstrated that vanilloids mediate apoptosis taking advantage of their lipophilicity using vanillic acid instead of capsaicin. In fact, using vanillic acid, which is water-soluble, no consequences on electron transport chain can be elicited (14, 15).

The fact that the induction of apoptosis by vanilloids does not depend on TRPV-1, but through other mechanisms may be demonstrated by experiments with capsazepine, an inhibitor of TRPV-1. If we observe prostate cancer cells in the presence of both capsaicin and capsazepine, we can see that the apoptotic role of capsaicin does not decrease: this is possible because while capsazepine inhibits TRPV-1, capsaicin continues to induce its apoptotic effect in a TRPV-1 non-dependent way (16).

Direct pathway leading to the vanilloid-mediated apoptosis is possible through two well known mechanisms: the delivery of reactive oxygen species (ROS) generated by NADH-oxidoreductase inhibition (existing in alteration of electron transport chain) and the interaction with caspases.

Inhibition of NADH-oxidoreductase

The NADH-oxidoreductase is a key enzyme in the electron transport chain, in which it represents the primary complex (complex I). In this chain, a great number of electrons fluently run towards a crescent reduction potential. The lower potential of reduction is

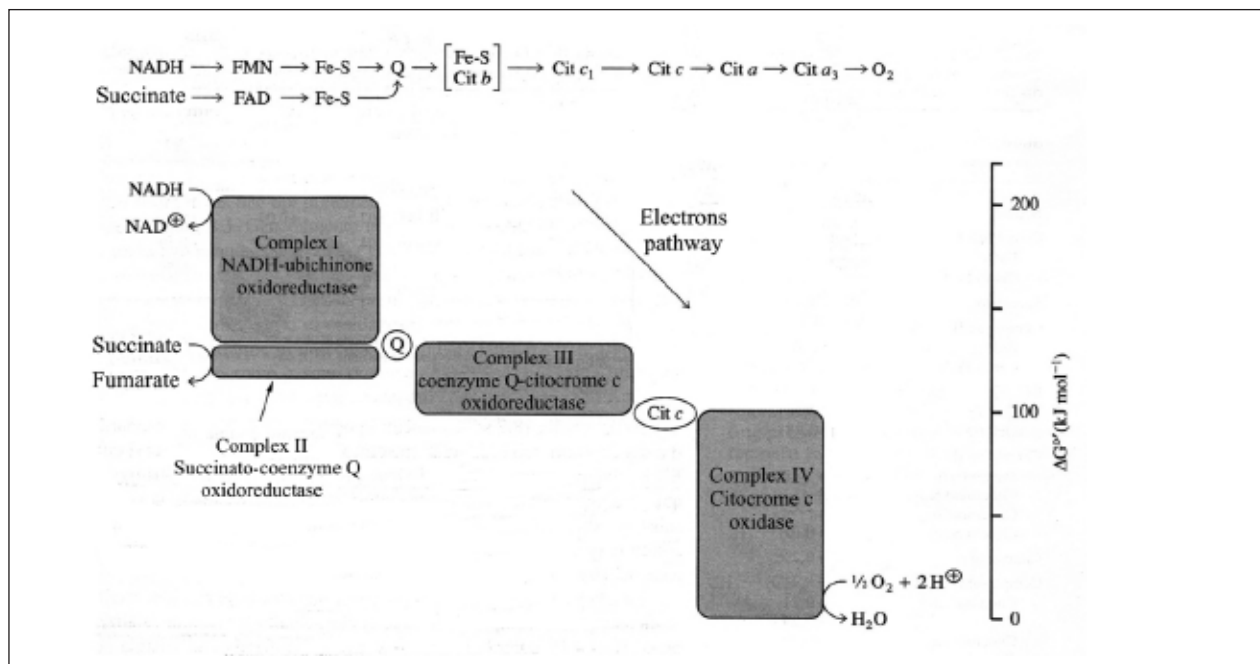


Figure 5. Electrons run in the electron transport chain

contained in NADH, while the higher potential is contained in the terminal oxidant agent: O₂. (Fig. 5).

The NADH-oxidoreductase gives two electrons to coenzyme Q as hydride ions H⁻. The reaction proceeds in two steps: in the first step the electrons are given to flavin-mono-nucleotide (FMN), becoming FMNH₂; in the second one the electrons are accepted by coenzyme Q, becoming QH₂ (Fig. 6, 7).

The vanilloids, especially capsaicin, realize their activity in this second step: particularly, capsaicin, dihydrocapsaicin and resiniferatoxin (ultrapotent analogue of capsaicin, extractable from *Euphorbia resinifera*

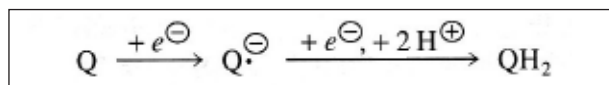


Figure 6. The role of the coenzyme Q in the electron transport chain

[17]), may be considered as coenzyme Q antagonists: in fact, they inhibit the run of the electrons through the electron transport chain, determining an excess of ROS (18).

Particularly, they determine an excess of super-oxid (·O₂⁻), hydroperoxid (H₂O₂) and hydroxilic ion (OH⁻).

Moreover, they are responsible for the redox instability of the cell and modify the Ca²⁺ membrane permeability (19). The role of the vanilloids in this mechanism may be demonstrated considering that their inhibition *in vitro* restores the correct run of the electron through the electron transport chain (19). Besides determining the intracellular Ca²⁺ growth, the reactive oxygen species are also responsible for an indirect DNA damage, since the loss of cell functioning induces apoptosis (19).

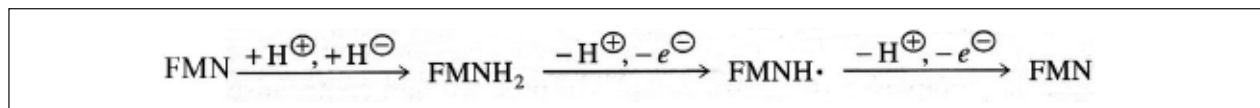


Figure 7. The role of the flavin-mono-nucleotide in the electron transport chain

Interaction with caspases

The interaction between vanilloid and caspases may be demonstrated in a plethora of tumoral cell lines and has been particularly studied in liver cancer and in epithelial breast cancer: in these cells, capsaicin reportedly increases caspasic activity (20). The activity of vanilloids is more evident in the phase of synthesis of the cell life (phase S), but it can also be observed in the phase G_0/G_1 , although it is less evident.

In the tumoral cells, apoptosis is inhibited by a caspase inhibitor: the most studied inhibitors belong to DEVD group (20). Capsaicin has a DEVD-asic activity: in fact the DEVD-mediated inhibition of apoptosis is not possible in presence of capsaicin. Capsaicin is thus capable to restore the mechanism of apoptosis generally lost in tumoral cell transformation (19, 20). The inhibitors belonging to the DEVD group interact with caspase 3 in the majority of cells; recently, other similar inhibitors have been discovered: they belong to the Z-YVAD group of proteins and preferentially interact with caspase 1. We acquired from literature that some vanilloids, such as capsaicin, inhibit caspase 3, while others, such as phorbol-12-myristate-13-acetate, preferentially interact with caspase 1 (21).

Phorbol-12-myristate-13-acetate, moreover, deserves particular consideration, because it can induce cell death also without caspase activation: because of this,

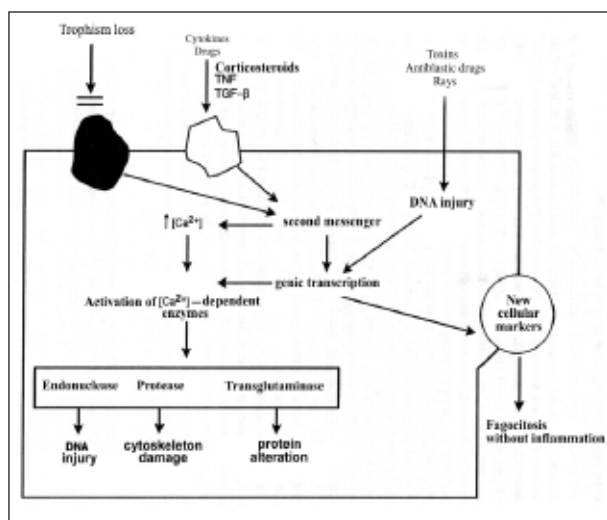


Figure 8. A generic mechanism of apoptosis

its activity may be considered not only pro-apoptotic, but also pro-necrotic (22, 21).

Direct pro-apoptotic activity of the vanilloids is selective for tumoral cells

The mechanisms of apoptosis described are potentially active in every cell, either tumoral or normal. The vanilloid-induced apoptosis, on the contrary, is most evident in tumoral cells. This has been reported by *Macho et al*, who made capsaicin interact with either tumoral or normal cells in two different cell cultures (15). In tumoral or activated cells, the interaction with capsaicin determines an important growth of ROS and a rapid increase in mitochondrial $\Delta\Psi$ (transmembrane potential). *Macho et al* observed that this does not happen in normal cells. In fact, it is a common agreement in literature that a long contact between normal cells and capsaicin does not induce precocious events of apoptosis, such as vacuolization or DNA damage, while a brief contact between tumoral cells and capsaicin induces the appearance of precocious events of cellular death (15).

Tumoral cells are known to present a non-controlled cellular activity. In order to make this anomalous activity possible, they need to have a high quantity of energy at their disposal. In these cells, the electron transport chain is constitutionally activated. For this reason, the delivery of ROS is higher than in normal cells: the interaction with capsaicin, which antagonizes the coenzyme Q_1 inhibits the run of the electron transport chain from complex I to complex II and allows a higher growth of reactive oxygen species in tumoral cells than in normal ones.

Another possible hypothesis is that of a greater sensibility of the electron transport chain complexes of the tumoral cells in comparison with the complexes of the normal cells (18): however, there is not a wide agreement in literature about this hypothesis.

Indirect pathway leading to vanilloid-mediated apoptosis in prostate cancer cells

In order to study the pro-apoptotic activity induced in prostate cancer cells by vanilloids, three cell

lines must be considered: normal prostate cells (RWPE-1), androgen-dependent prostate cancer cells deriving from lymph nodes (LNCaP) and androgen-independent prostate cancer cells deriving from bone metastasis (PC-3).

TRPV-1, the receptor for vanilloids, is expressed in LNCaP and PC-3 cells only, and not in RWPE-1 cells. It is encoded by a 2514 nucleotides DNA track, which expresses a 838 aminoacid protein, whose molecular mass is 95 KDa in the non-glycosilated state and 114 KDa after glycosilation (23).

This receptor has been found not only on the plasmatic membrane of the epithelial prostate cells, but also on periprostatic nervous bundle cells and on the interstitial cells of the prostatic stroma (24, 25).

On the same cells, another receptor is expressed, belonging to the same family of TRPV-1: this receptor is TRPV-6. It is placed on the plasmatic membrane and it generates a transient Ca^{2+} inward current (26, 27).

In the different cells of the majority of tissues, a stimulation or the simple cellular activity determines a mobilization of the intracellular calcium $[\text{Ca}^{2+}]_i$ in the citosol; afterwards, the plasmatic membrane channels for Ca^{2+} are opened and a store-operated release of Ca^{2+} from internal stores induces sustained Ca^{2+} influx. The concentration of calcium in the interstitial space is higher than in the intracellular space: this determines a Ca^{2+} influx at the only opening of Ca^{2+} channels. This current is named I_{SOC} (26).

In the cells expressing TRPV-6, a similar current is present, which is called $I_{\text{TRPV-6}}$. The differences between I_{SOC} and $I_{\text{TRPV-6}}$ concern quantitative characteristics (in fact, the number of Ca^{2+} ions entering in the cell thanks to $I_{\text{TRPV-6}}$ is lower than the one entering thanks to I_{SOC}) and channel regulation aspects.

In addition, TRPV-6 responds to androgens, to anti-androgens and to 1-25-dihydroxyvitamin D3.

Nevertheless, TRPV-6 Ca^{2+} growth is not sufficient to start any pro-apoptotic pathway because it is too low. This is the reason why TRPV-6, unlike TRPV-1, does not have a role in apoptosis inductions. Some molecules, moreover, such as carbacole, determine the only mobilization of calcium from intracellular stores. This, too, is not important in apoptosis induction (28). The interaction of capsaicin or other

vanilloids with TRPV-1, on the contrary, is responsible for the mobilization of calcium from the interstitial space, where its concentration is higher, to the intracellular space, where its concentration is normally lower (27).

The importance of calcium in a generic mechanism of apoptosis is well exposed in figure 8. TRPV-1 is directly involved in an important increase in intracellular calcium, unlike TRPV-6, which only generates little Ca^{2+} growth. As evidence of the role of the vanilloid-TRPV-1 interaction in determining the intracellular Ca^{2+} growth, we can confirm that no $[\text{Ca}^{2+}]_i$ modification may be shown in cells TRPV-1⁻ (not expressing the receptor) (28).

The first evidence of apoptosis in LNCaP and PC-3 cells as a result of $[\text{Ca}^{2+}]_i$ is the expression of a great number of phosphatidyl-serine on the external layer of the plasma membrane. Phosphatidyl-serine expression is completely caspase-independent, as *Jambirina et al* demonstrated: in fact, after DEVD-group molecules overexpression, in literature an increase in phosphatidyl-serine on the external layer of plasma membrane as a consequence of the prolonged interaction with vanilloids is reported.

As we have already explained, DEVD group molecules inhibit the pathway leading to apoptosis, directly interacting with caspases. The inhibition of caspases, thanks to DEVD-molecules, determines the overexpression of phosphatidyl-serine as a consequence of the TRPV-1-capsaicin interaction.

A second effect of the interaction between the vanilloids and TRPV-1 receptor may be reported: it is the fast decrease of the transmembrane mitochondrial potential ($\Delta\Psi_m$). It begins 2-3 minutes after the interaction with the capsaicin and reaches a plateau after 10-15 minutes of incubation (16, 22). After about 10 hours of incubation, a second peak of $\Delta\Psi_m$ decrease is reported. The first peak of $\Delta\Psi_m$ decrease is determined by the alteration induced in the plasmatic membrane, while the second peak is determined by the alteration induced in the mitochondrial membrane (23). This may be demonstrated incubating with both capsaicin or electron transport chain inhibitors, such as ruthenium (which inhibits the complex I of the electron transport chain) and potassium cyanide (which inhibits complex III). As a consequence of the double

incubation, the electron transport chain is completely inhibited and only the first peak of $\Delta\Psi_m$ decrease is reported (16).

The fast $\Delta\Psi_m$ decrease leads the cell to the energetic disarrangement: in a short time it collapses and the cellular program of apoptosis starts. A plethora of evidences can be immediately revealed: chromatin condensation, cytoskeleton damage and cross links between intracellular proteins may be observed.

Conclusions

The vanilloids, such as the capsaicin and its ultrapotent analogue resiniferatoxin, lead the cell to apoptosis through a direct pathway (receptor-independent pathway) and an indirect pathway (receptor-dependent). In the first case, they inhibit the electron transport chain because they are analogue of the coenzyme Q. As a result of the electron transport chain inhibition, a great delivery of reactive oxygen species (ROS) and consequent cell damage and apoptosis mechanism activation may be reported. Through the direct pathway, the vanilloids induce apoptosis by interacting with caspases, particularly caspase 1 and 3.

The receptor-dependent pathway (indirect pathway), on the contrary, needs the interaction with TRPV-1, a receptor containing a ionotropic channel selective for calcium. The interaction with the receptor leads to the intracellular calcium growth and consequently to the evidence of precocious and late elements of apoptosis.

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