BYPASSING THE EXECUTIONER: CASPASE-INDEPENDENT CELL DEATH PATHWAYS ASSOCIATED WITH PIPERACEAE PHYTOCHEMICALS

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ABSTRACT

Apoptosis is commonly associated with the activation of caspases, a family of proteases involved in the execution of programmed cell death. However, cancer cells may also undergo cell death through pathways that do not depend on caspase activation. Mitochondrial factors such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG) are important mediators of these alternative pathways. Phytochemicals obtained from plants belonging to the family Piperaceae have attracted attention because of their observed cytotoxic and antitumour properties.

Objective: *This article explores experimental evidence concerning the involvement of caspase-independent cell death mechanisms in the anticancer activity of selected Piperaceae-derived extracts and phytochemicals, with particular emphasis on Piper longum.*

Discussion: *Experimental findings from Piper longum fruit extract provide evidence of caspase-independent cell death in cancer cells. The extract was associated with mitochondrial membrane disruption, increased reactive oxygen species (ROS), and the release of AIF and EndoG. Importantly, inhibition of caspases did not prevent the reduction in cancer-cell viability. Other investigations involving piperlongumine, piperine, and piplartine have demonstrated cytotoxic or pro-apoptotic activities. These findings do not independently establish the involvement of AIF, EndoG, or other caspase-independent pathways. The available evidence therefore indicates that the mechanistic understanding of Piperaceae-derived compounds remains uneven, with substantially stronger direct evidence for the ethanolic fruit extract of Piper longum than for other compounds and species.*

Conclusion: *Caspase-independent cell death represents a very important mechanism associated with the anticancer activity of Piper longum fruit extract. Future investigations using pathway-specific molecular interventions, appropriate non-malignant cellular controls, and reproducible phytochemical characterisation are going to be helpful in establishing the mechanisms and therapeutic relevance of individual Piperaceae constituents.*

Keywords: *Piper longum, Piperaceae, caspase-independent cell death, cancer cells.*

INTRODUCTION

Cancer is a major global health challenge, and the development of effective therapeutic strategies is an important area of biomedical research. Although conventional anticancer treatments can suppress tumour growth, their effectiveness may be limited by toxicity, treatment resistance, and the ability of malignant cells to evade programmed cell death. Consequently, identifying alternative mechanisms capable of inducing cancer-cell death has become an important research objective.

Apoptosis is a regulated form of cell death characterised by distinct biochemical and morphological changes, including chromatin condensation, DNA fragmentation, membrane alterations, and cellular disintegration. In the classical apoptotic pathway, activation of initiator and executioner caspases contributes to the controlled destruction of cellular components. However, cell death is not exclusively dependent on caspase activation. Experimental investigations have demonstrated that cells may undergo regulated death even when caspase activity is inhibited (Kroemer & Martin, 2005).

Mitochondria play a central role in several cell-death pathways. In addition to releasing cytochrome c, mitochondria can release apoptosis-inducing factor (AIF) and endonuclease G (EndoG). These proteins can participate in nuclear changes associated with cell death independently of the conventional caspase execution pathway. AIF was initially characterised as a mitochondrial protein capable of inducing nuclear changes associated with apoptosis (Susin et al., 1999).

Natural products have attracted considerable attention as potential sources of anticancer compounds. Plants belonging to the family Piperaceae contain several biologically active constituents, including alkaloids and amides. *Piper longum*, commonly known as long pepper, is particularly recognised for its phytochemical constituents, including piperine and piperlongumine. Experimental studies have investigated the cytotoxic and antitumour properties of extracts and isolated constituents from this plant.

An important experimental investigation by Ovadje et al. (2014) demonstrated that an ethanolic extract of *Piper longum* fruit could induce caspase-independent cell death in selected cancer-cell models. The findings implicated mitochondrial dysfunction, ROS generation, and the release of AIF and EndoG. These observations

provide a specific mechanistic basis for investigating the relationship between Piperaceae-derived compounds and alternative cell-death pathways.

Nevertheless, cytotoxicity, conventional apoptosis, and caspase-independent cell death are distinct experimental outcomes. A reduction in cell viability does not automatically establish the activation of a particular cell-death mechanism. Therefore, the available experimental findings must be interpreted according to the molecular evidence actually demonstrated.

BIOLOGICAL BASIS OF CASPASE-INDEPENDENT CELL DEATH

Conventional Caspase-Dependent Apoptosis

The conventional apoptotic pathway involves a coordinated sequence of molecular events regulated by several intracellular proteins. The intrinsic pathway is commonly initiated by cellular stress, DNA damage, oxidative injury, or other intracellular disturbances. These signals can alter mitochondrial membrane integrity and promote the release of pro-apoptotic factors.

Cytochrome c released from mitochondria interacts with apoptotic protease-activating factor 1 (Apaf-1), resulting in apoptosome formation and subsequent activation of caspase-9. Caspase-9 then activates executioner caspases, particularly caspase-3 and caspase-7. These enzymes cleave several cellular proteins and contribute to the characteristic morphological and biochemical changes associated with apoptosis.

Although this pathway is well established, caspase activation is not an absolute requirement for every form of programmed cell death. In certain experimental conditions, cellular death can continue despite the inhibition of caspase activity (Kroemer & Martin, 2005).

Apoptosis-Inducing Factor (AIF)

Apoptosis-inducing factor is a mitochondrial flavoprotein that participates in caspase-independent cell-death processes. Under normal conditions, AIF is predominantly located in the mitochondrial intermembrane space. Following appropriate cellular stress and mitochondrial membrane disruption, AIF can be released into the cytoplasm and subsequently translocate to the nucleus.

Nuclear AIF is associated with chromatin condensation and large-scale DNA fragmentation. Unlike conventional executioner caspases, AIF can contribute to nuclear changes without requiring the activation of the classical caspase cascade.

The original characterisation of AIF established its role as a mitochondrial factor capable of inducing nuclear changes associated with apoptosis (Susin et al., 1999). Its involvement is particularly relevant when cell death persists despite pharmacological inhibition of caspases.

Endonuclease G (EndoG)

Endonuclease G is another mitochondrial protein with caspase-independent DNA degradation. Under physiological conditions, it is located in mitochondria and participates in mitochondrial nucleic-acid processing. Following mitochondrial disruption, EndoG may be released and translocate to the nucleus, where it can contribute to DNA degradation. Its activity provides an additional mechanism through which mitochondrial injury may lead to nuclear damage without depending entirely on caspase activation.

The simultaneous investigation of AIF and EndoG is useful when evaluating whether a plant-derived preparation induces mitochondrial cell-death mechanisms.

Mitochondrial Dysfunction and Oxidative Stress

Mitochondria regulate cellular energy production and participate in the maintenance of intracellular redox balance. Excessive production of reactive oxygen species can damage mitochondrial components, disrupt membrane integrity, and alter cellular metabolic functions.

Oxidative stress may appear to contribute to the initiation of cell-death pathways through several mechanisms. These include mitochondrial membrane permeabilisation, the release of pro-death proteins, and changes in the expression or activity of proteins regulating cell survival.

However, ROS generation alone does not establish caspase-independent apoptosis. To demonstrate a specific pathway, oxidative changes must be evaluated alongside additional molecular indicators, such as caspase inhibition, mitochondrial membrane changes, and the release or nuclear translocation of AIF and EndoG.

Distinction Between Caspase-Independent Death and General Cytotoxicity

An important consideration in interpreting phytochemical studies is the distinction between general cytotoxicity and mechanistically established caspase-independent cell death.

A compound may reduce cell viability through several possible mechanisms, including cell-cycle arrest, oxidative injury, membrane disruption, necrosis, or conventional apoptosis. So, cell viability assays alone cannot establish the exact mechanism responsible for the observed effect.

Evidence supporting caspase-independent cell death becomes stronger when investigations demonstrate that:

1. Cell death persists despite appropriate inhibition of caspase activity.
2. Mitochondrial membrane integrity is disrupted.

3. AIF or EndoG is released from mitochondria or translocated to the nucleus.
4. Nuclear changes or DNA degradation are consistent with the proposed pathway.
5. Appropriate experimental controls exclude alternative explanations.

EXPERIMENTAL EVIDENCE FROM PIPERACEAE-DERIVED PREPARATIONS

Ethanolic Fruit Extract of *Piper longum*

One of the most directly relevant experimental investigations was conducted by Ovadje et al. (2014), who examined the anticancer activity and biochemical mechanism of an ethanolic extract of *Piper longum* fruit, designated PLX.

The researcher evaluated the effects of the extract using several cancer-cell models and non-cancerous cellular controls. The study examined cell viability, oxidative stress, mitochondrial membrane potential, caspase activity, and the release of mitochondrial proteins linked with cell death.

The findings demonstrated that PLX reduced cancer-cell viability and induced cell death in a dose- and time-dependent manner. The observed effects were associated with increased ROS production and disruption of mitochondrial membrane potential.

A particularly important aspect of the investigation was the use of the pan-caspase inhibitor Z-VAD-fmk. Cancer cells were pretreated with the inhibitor before exposure to PLX. The inhibition of caspase activity did not prevent the reduction in cell viability, indicating that the cytotoxic effect was not dependent on conventional caspase activation.

The researcher also examined the involvement of mitochondrial proteins. Their findings indicated the release of AIF and EndoG from isolated mitochondria following exposure to the extract. These observations supported the involvement of mitochondrial factors in the cell-death process.

The study further observed that the extract could reduce the growth of colon cancer tumours in an experimental mouse model. This finding provided additional evidence of biological activity in vivo. However, the animal findings should not be interpreted as proof of clinical effectiveness or as confirmation that the same molecular pathway was responsible for all observed antitumour effects.

The importance of this investigation lies in the combination of functional and molecular observations. Rather than relying solely on cell viability measurements, the researchers examined caspase inhibition, mitochondrial dysfunction, oxidative stress, and the involvement of AIF and EndoG.

These findings provide direct experimental support for caspase-independent cell death associated with an ethanolic fruit extract of *Piper longum* (Ovadje et al., 2014).

Piperlongumine and Oxidative Stress-Associated Apoptosis

Piperlongumine is a naturally occurring amide alkaloid obtained from *Piper longum*. Its biological activity has been investigated in several cancer-cell models, particularly because of its observed effects on cellular proliferation, oxidative stress, and apoptosis.

Gong et al. (2014) investigated the effects of piperlongumine on human ovarian cancer cells. Their findings demonstrated that piperlongumine inhibited cancer-cell growth, induced apoptosis, and caused G2/M cell-cycle arrest. The researcher also observed increased intracellular ROS levels following treatment.

Importantly, pretreatment with the antioxidant N-acetylcysteine reversed ROS accumulation and the observed apoptotic effects. The study also demonstrated synergistic growth-inhibitory effects when piperlongumine was combined with cisplatin or paclitaxel under the experimental conditions.

These findings support an association between oxidative stress and piperlongumine-induced apoptosis in ovarian cancer cells. However, the study does not independently establish that the observed cell death occurred through an AIF- or EndoG-mediated caspase-independent pathway.

Consequently, the findings of Gong et al. (2014) should be interpreted as evidence of ROS-associated apoptosis rather than direct confirmation of caspase-independent cell death.

This distinction is important because piperlongumine-induced apoptosis may involve conventional apoptotic signalling, and the involvement of ROS does not necessarily indicate that caspases are dispensable.

Piperine and Antitumour Activity

Piperine is an alkaloid found in several *Piper* species, including *Piper longum* and *Piper nigrum*. It has been investigated for various biological activities, including cytotoxic and immunomodulatory effects.

Sunila and Kuttan (2004) examined the immunomodulatory and antitumour activities of *Piper longum* fruit extract and piperine. Their investigation demonstrated cytotoxic effects against Dalton's lymphoma ascites and Ehrlich ascites carcinoma cells. The study also observed antitumour effects in experimental animal models.

The researcher observed changes in immunological parameters following administration of the extract and piperine, suggesting that their biological effects were not restricted to direct cytotoxicity.

However, the study did not establish the involvement of AIF, EndoG, or caspase-independent mitochondrial cell-death signalling. Therefore, the findings support the antitumour and immunomodulatory potential of *Piper longum* extract and piperine but cannot be used as direct evidence of caspase-independent apoptosis.

The distinction is particularly important when comparing crude plant extracts with isolated phytochemical constituents. The biological activity of an extract may result from multiple constituents acting together, whereas the effects of an isolated compound may differ in potency and mechanism.

Piplartine and Antiproliferative Activity

Piplartine, also known as piperlongumine, is a bioactive amide associated with *Piper* species. It has attracted interest because of its observed antiproliferative and cytotoxic properties.

Bezerra et al. (2005) investigated the cytotoxic activities of piperine and piplartine using several experimental systems, including tumour-cell lines. Their findings demonstrated substantial antiproliferative activity, with piplartine showing greater activity than piperine in the MTT-based tumour-cell assays used in that investigation. The study provided evidence that these compounds possess biological activities capable of affecting cellular proliferation and survival. However, the investigation did not establish the involvement of AIF, EndoG, or other specific caspase-independent cell-death mechanisms.

Accordingly, the results of Bezerra et al. (2005) support the cytotoxic potential of piplartine and piperine but do not establish their participation in the mitochondrial pathway described in the *Piper longum* fruit-extract investigation.

COMPARATIVE ASSESSMENT OF THE EXPERIMENTAL FINDINGS

The available experimental investigations demonstrate that Piperaceae-derived preparations and constituents can influence cancer-cell survival. However, the strength of the mechanistic evidence differs considerably between studies.

The following table distinguishes direct evidence of caspase-independent cell death from findings limited to cytotoxicity or conventional apoptosis.

Table 1. Experimental evidence concerning the anticancer activity of selected Piperaceae-derived preparations

Plant preparation or constituent	Experimental findings	Evidence for caspase-independent cell death
Ethanolic *Piper longum* fruit extract (PLX)	Reduced cancer-cell viability, increased ROS, mitochondrial membrane disruption, caspase-independent effects, and release of AIF and EndoG	Direct experimental evidence observed
Piperlongumine	ROS accumulation, apoptosis, G2/M arrest, and enhanced effects with cisplatin or paclitaxel in ovarian cancer cells	Not established by the cited ovarian-cancer investigation
Piperine	Cytotoxic and antitumour effects in experimental cellular and animal models	Not established
Piplartine	Antiproliferative activity in experimental tumour-cell assays	Not established

Note. The table summarises the findings of Ovadje et al. (2014), Gong et al. (2014), Sunila and Kuttan (2004), and Bezerra et al. (2005).

Principal Findings

The strongest direct evidence for caspase-independent cell death among the experimental investigations considered here comes from the study of ethanolic *Piper longum* fruit extract conducted by Ovadje et al. (2014). Three observations are particularly important.

First, the reduction in cancer-cell viability persisted despite the use of a pan-caspase inhibitor. This finding indicates that conventional caspase activation was not essential for the observed cytotoxic effect under the experimental conditions.

Second, the researcher observed mitochondrial membrane disruption and increased ROS production. These findings suggest that mitochondrial dysfunction was associated with the biological activity of the extract.

Third, the release of AIF and EndoG provided molecular evidence supporting the involvement of mitochondrial factors associated with caspase-independent cell death.

In comparison, investigations involving piperlongumine, piperine, and piplartine demonstrate important cytotoxic and antiproliferative activities but provide less direct evidence concerning the specific pathway responsible for cell death.

Importance of Mitochondrial Pathways

The mitochondrial pathway is particularly relevant because mitochondria contain several proteins capable of influencing cell survival and death.

The release of AIF and EndoG provides an alternative mechanism for nuclear changes and DNA degradation when conventional caspase activation is inhibited. The findings observed by Ovadje et al. (2014) therefore suggest that *Piper longum* fruit extract may influence cancer-cell survival through mitochondrial mechanisms that extend beyond conventional caspase-dependent apoptosis.

But, the detection of AIF and EndoG release should not be interpreted as proof that each protein independently accounts for the entire cytotoxic effect. Further experiments involving targeted inhibition, genetic knockdown, or other pathway-specific interventions would be necessary to establish their individual contributions.

Role of Oxidative Stress

ROS generation is another important finding in investigations of *Piper longum*-derived preparations and piperlongumine.

Oxidative stress can influence mitochondrial function, cellular signalling, and the regulation of cell-death pathways. The study by Ovadje et al. (2014) associated increased ROS with mitochondrial dysfunction following exposure to PLX.

Gong et al. (2014) also demonstrated ROS-associated apoptosis following piperlongumine treatment in ovarian cancer cells. However, the response to antioxidant pretreatment in that study indicates that ROS contributed to the observed apoptotic effects.

The two investigations therefore support the importance of oxidative stress but do not demonstrate identical mechanisms. Differences in the experimental preparation, cellular model, and molecular endpoints must be considered when interpreting the results.

LIMITATIONS OF THE AVAILABLE EXPERIMENTAL EVIDENCE

Direct experimental evidence concerning caspase-independent cell death is limited compared with the number of studies reporting general cytotoxic or apoptotic effects.

The biological activity of a plant extract may vary according to geographical origin, environmental conditions, plant maturity, harvesting procedures, storage, and extraction methods.

Cancer-cell lines differ in their genetic characteristics, metabolic activity, antioxidant capacity, and sensitivity to cell-death signals.

CONCLUSION

Experimental investigations indicate that selected Piperaceae-derived preparations and phytochemicals possess cytotoxic, antiproliferative, and antitumour properties. Among the studies considered, the investigation of ethanolic *Piper longum* fruit extract by Ovadje et al. (2014) provides the clearest direct evidence of caspase-independent cell death.

The observed persistence of cytotoxicity despite caspase inhibition, together with mitochondrial membrane disruption and the release of AIF and EndoG, supports the involvement of an alternative mitochondrial cell-death pathway.

Other investigations involving piperlongumine, piperine, and piplartine demonstrate biological activity but do not establish the same molecular mechanism. Their cytotoxic and apoptotic effects should therefore be interpreted according to the specific experimental findings rather than automatically attributed to caspase-independent pathways.

Overall, the available evidence identifies *Piper longum* fruit extract as a promising subject for further mechanistic investigation. However, additional studies involving standardised preparations, pathway-specific molecular experiments, appropriate cellular controls, and in vivo validation are required to establish the precise mechanisms and potential therapeutic relevance of Piperaceae-derived compounds.

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