

# Natural products as inducers of apoptosis: Implication for cancer therapy and prevention

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**Apoptosis is a programmed cell death and a highly organized physiological mechanism to destroy injured or abnormal cells. Apoptotic cells exhibit remarkable morphological features and characteristic molecular expression. Apart from physiological stimuli there are exogenous factors which can contribute to induce apoptosis. The induction of apoptosis in tumour cells is considered very useful in the management and therapy as well as in the prevention of cancer. A wide variety of natural substances have been recognized to have the ability to induce apoptosis in various tumour cells of human origin. These substances are compounds with different chemical entities and many of these are present in plants with medicinal value and in various fruits and vegetables commonly consumed by humans. Mode of action in the apoptotic pathway of some of these compounds has been delineated.**

APOPTOSIS or programmed cell death is a highly organized physiological process to eliminate damaged or abnormal cells. It also plays a major role in embryogenesis where apparently normal cells undergo apoptosis. It is involved in maintaining homeostasis in multicellular organisms. The outstanding feature of apoptosis is its remarkable stereotyped morphology showing condensation of nuclear heterochromatin, cell shrinkage and loss of positional organization of organelles in the cytoplasm<sup>1,2</sup>. Although morphological characteristics initially described apoptosis, it is now clear that there is a highly complex molecular process involved. Possible convergence of various events results in the activation of the cellular machinery responsible for apoptosis. The p53 gene which is strongly implicated in animal and human carcinogenesis is a significant regulator of the process of apoptosis<sup>3</sup>. The p53 mutations are now recognized to be the most common genetic changes in human cancers and p53 acts as a tumour suppressor gene. While apoptotic pathway is related to induction of p53, this pathway is held in check by the anti-apoptotic gene *bcl-2* (ref. 4). The proto-oncogene *bax* forms a heterodimer with *bcl-2* and accelerates the process of apoptosis. Activation of transcription factor NF- $\kappa$ B involving its translocation to the nucleus has been linked to apoptosis<sup>5</sup>. It can activate both the apoptotic and anti-apoptotic genes.

The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligonucleosomal fragments. This results from inter-nucleosomal chromatin cleavage by endogenous endonucleases in multiples of 180 base pairs<sup>6</sup>. This fragmentation is regarded as the hallmark of apoptosis. In cells undergoing apoptosis there is activation of a family of proteases called caspases, so named because they have an obligatory cysteine residue within the active site and cleave peptides adjacent to an aspartic acid residue<sup>7</sup>. Activation of caspases appears to be directly responsible for many of the molecular and structural changes in apoptosis. These include degradation of DNA repair enzyme poly(ADP)ribosepolymerase (PARP)<sup>8</sup> and DNA-dependent protein kinase (DNA-PK)<sup>9</sup>, and cleavage of chromatin at inter-nucleosomal sites mediated by caspase-activated DNase (CAD)<sup>10</sup>. Cleavage of cytoskeletal elements and membrane proteins by calpains (Ca<sup>2+</sup>-binding and thiol-containing proteins) may partly explain the fragmentation of the cells to multiple, spherical 'apoptotic bodies'. These bodies are typically phagocytocysed by adjacent cells or macrophages<sup>11</sup> (Figure 1).

## Need for apoptotic inducers

The accepted modality for cancer treatment involves surgery, radiation and drugs, singly or in combination. Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally, cures. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis. Thus, modulating apoptosis may be useful in the management and therapy or prevention of cancer. Synthesis or modification of known drugs continues as an important aspect of research. However, a vast amount of synthetic work has contributed relatively small improvements over the prototype drugs. There is a continued need for new prototypes – new templates to use in the design of potential chemotherapeutic agents. Significantly, natural products are providing such templates.

Recent studies on tumour inhibitory compounds of plant origin have yielded an impressive array of novel

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structures. Besides, epidemiological studies suggest that consumption of diets containing fruits and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer<sup>12</sup>. Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells<sup>13-15</sup>. It has become increasingly evident that apoptosis is an important mode of action for many anti-tumor agents, including ionizing radiation<sup>16</sup>, alkylating agents such as cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)<sup>17</sup>, topoisomerase inhibitor etoposide<sup>18</sup>, cytokine tumour necrosis factor (TNF)<sup>19</sup>, taxol<sup>20</sup>, and N-substituted benzamides such as metoclopramide and 3-chloroprocainamide<sup>21</sup>. Apoptotic induction has been a new target for innovative mechanism-based drug discovery<sup>22,23</sup>.

It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. Evidence has emerged from various studies that suggest that products derived from plants are useful in the treatment as well as in the prevention of cancer. Chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but, their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention. Understanding the modes of action of these compounds should provide useful informa-

tion for their possible application in cancer prevention and perhaps also in cancer therapy.

### Plant extracts used as traditional medicine

*Selaginella tamariscina* (Beauv.), with the popular Korean name 'Keoun Back', is a traditional medicinal plant for therapy of advanced cancer patients in the Orient which has been shown to modify gene expression and cytokine production<sup>24</sup>. Lee *et al.*<sup>25</sup> reported that water extract of this plant efficiently increased p53 gene expression and induced G1 arrest and suggested that this might contribute to cytotoxic effects by causing apoptotic DNA fragmentation in human leukaemia cell line U-937 and human ovarian cancer cell line A-2780. Hot water extract of the bark of Nikko maple (*Acer nikoense*), a traditional crude drug, has been known for its cytotoxicity (mediated through apoptosis) in susceptible and resistant mouse leukaemia P-388 cell lines, and this extract also increased the expression of sialylated glycoconjugates in apoptotic cells<sup>26</sup>. *Uncaria tomentosa* (Willd.) DC, also known as 'Uña de gato' or 'Cat's claw', has been used in South American traditional medicine. The native Indians particularly of the Amazon region use tea made of the bark or roots for the treatment of a variety of health disorders including cancer, arthritis and infectious diseases. Organic solvent extracts of this plant were shown to have cytostatic, contraceptive and anti-inflammatory activity<sup>27</sup>. Sheng *et al.*<sup>28</sup> reported that water extract of this plant induced apoptosis-mediated cytotoxicity in human leukaemic cell lines HL-60, K-562 and human EBV-transformed B-lymphoma cell line Raji.

Extracts of *Solanum muricatum* (Pepino) have been shown to induce apoptotic cell death in prostate (PC-3, DUI-45), stomach (MKN-45), liver (QSY-7721, SK-HEP-1), breast (MDA-MB-435), ovarian (OVCAR), colon (HT-29) and lung (NCI-H-209) cancer cells and some normal (NHP, HUVEC, WI-38) cells in culture. This plant extract also induced DNA ladder formation and PARP cleavage<sup>29</sup>. *Alpinia oxyphylla* Miquel (Zingiberaceae) used in traditional Oriental herbal medicine has been shown to induce apoptosis-mediated cytotoxicity in HL-60 cells in culture<sup>30</sup>. Unpublished observations from the authors' laboratory have revealed that ethanolic extracts of roots and leaves of *Tiliacora racemosa* Colebr. and the Ayurvedic oil preparation from *Semecarpus anacardium* Linn. nuts possess cytotoxic activity towards cell lines K-562 and HL-60. It is noteworthy that these plants which are used in traditional Indian medicine induce cell death via apoptosis. Extract from *Viscum album* L. (Loranthaceae), European mistletoe, which is one of the most widely used alternative cancer therapies in Europe, has been recognized to induce cytotoxicity in human lymphocytes in culture, probably through apoptosis<sup>31-33</sup>. Mistletoe preparations from the host trees *Pinus*, *Malus*,

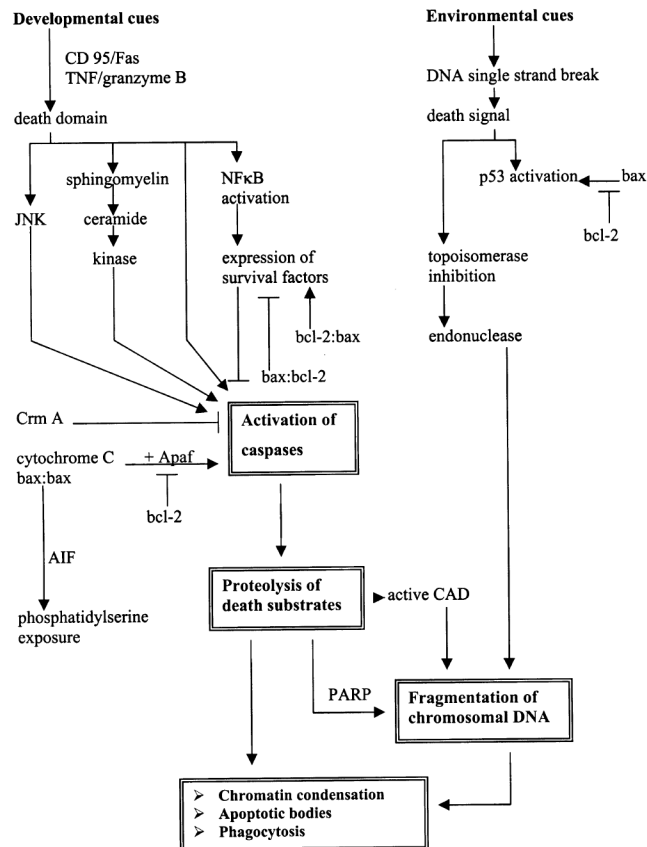


Figure 1. Pathways of apoptosis.

*Abies* and *Quercus*, when clinically applied have been shown to induce apoptosis and various cytokines in peripheral blood leukocytes<sup>34</sup>. Chinese herbal supplement PC-SPES has shown apoptosis and dose-dependent down-regulation of *bcl-6* in mutu 1 cells<sup>35</sup>. *Salvia miltiorrhiza* is a traditional Chinese herbal medicine commonly used to treat liver diseases in China for centuries. This plant extract exerted clear cytotoxic effects and strongly inhibited the proliferation of human hepatoma cell line, HepG(2) cells, through apoptosis<sup>36</sup>.

Water-soluble macromolecular components of *Artemisia capillaris* Thunberg exhibited inhibition of cell proliferation and apoptosis when studied on human hepatoma cell line (SMC-7721)<sup>37</sup>. The cytotoxic effect of conifer *Tetraclinis articulata* essential oil on a number of human cancer cell lines and peripheral blood lymphocytes is mediated through apoptosis<sup>38</sup>. *Phyllanthus orbicularis*, a plant of genus *Phyllanthus*, is used in Cuban traditional medicine for its antiviral activity against Hepatitis B virus and A and B flu virus. The aqueous extract of this plant induced apoptosis in Chinese hamster ovary (CHO) cells<sup>39</sup>. Seeds from *Acalypha wilkesiana* (Euphorbiaceae) are essential components of a complex plant mixture used empirically by traditional healers in south-west Nigeria to treat breast tumours and inflammation<sup>40</sup>. Bussing *et al.*<sup>40</sup> observed an induction of apoptosis and generation of reactive oxygen intermediates in granulocytes by an aqueous extract of the seeds. This extract induced the release of the pro-inflammatory cytokines TNF- $\alpha$  and interleukin-6 (IL-6) and also T-cell associated cytokines interleukin-5 (IL-5) and interferon-gamma (IFN- $\gamma$ ).

## Phenolic compounds

The human diet contains a complex mixture of plant polyphenols and it is believed that human individuals may consume as much as one gram of plant phenols per day in their diet<sup>41-43</sup>. Studies have shown that cytotoxic effect of these phenols against different tumours is mediated through apoptosis<sup>44</sup>. Gallic acid selectively induces cell death in various transformed cell lines such as PLC/PRF/5 (human hepatoma), HL-60 RG (human promyelocytic leukaemia), P-388 D1 (mouse lymphoid neoplasma), HeLa (human epithelial carcinoma), dRLh-84 (rat hepatoma) and KB (human epidermoid carcinoma).<sup>44</sup> This plant phenol which is a well-known natural antioxidant induced DNA fragmentation in four different human myelogenous leukaemic cell lines (HL-60, ML-1, U-937, THP-1), but not in human T-cell leukaemia (MOLT-4) and erythroleukaemic (K-562) cell lines. In contrast, tannic acid and caffeic acid induced DNA fragmentation only in HL-60 cells<sup>45</sup>. Gallic acid induced apoptosis in HL-60 RG cells through reactive oxygen species generation, Ca<sup>2+</sup> influx and activation of calmodulin<sup>44</sup>. Considering the ability of polyphenol as tannin to absorb proteins and metal ions, there is a possi-

bility that gallic acid can elicit death signals through some specific receptor or enzyme. Structure activity analysis revealed that methylation of phenolic hydroxyl groups and decarboxylation abolished the cytotoxic activity of gallic acid and suggested that the existence of three free adjacent phenolic hydroxyl groups and a carboxyl group, regardless of being free or modified, may be required to induce apoptosis<sup>44</sup>. The apoptosis-inducing activity of tannic acid and caffeic acid did not depend on extracellular Ca<sup>2+</sup>. Caffeic acid induced DNA fragmentation, whereas two other phenylpropanoid monomers, i.e. *p*-coumaric acid and ferulic acid were inactive. A dehydrogenation polymer of caffeic acid showed slightly lower activity than its corresponding monomer, but dehydrogenation polymers of *p*-coumaric acid and ferulic acid were inactive<sup>45</sup>. The active component of the folk medicine propolis, caffeic acid phenethyl ester (CAPE), displayed selective cytotoxicity toward oncogene-transformed cloned rat embryo fibroblast (CREF) cells through apoptosis<sup>46</sup>. It was also reported that *bcl-2* had a protective effect against CAPE-induced cell death<sup>15</sup>.

Curcumin, a phenolic compound that has been identified as the major pigment in turmeric, induces apoptosis in transformed rodent and human cells in culture<sup>14,47-50</sup>. However, it has no apparent effect on nontumorigenic rat embryo fibroblast cells, which makes it a useful adjunct to chemotherapy<sup>14,15</sup>. Curcumin mediated the chemopreventive action through inhibition of formation of cyclooxygenase (COX) metabolites, which could provide a mechanism for induction of apoptosis<sup>51,52</sup>. Yakuchinone A (1-[4'-hydroxy-3'-methoxyphenyl]-7-phenyl-3-heptanone) and yakuchinone B (1-[4'-hydroxy-3'-methoxyphenyl]-7-phenylhept-1-en-3-one), which are present in *A. Oxyphylla*, having a diarylheptanoid moiety with a carbonyl functional group have been reported to have apoptotic inducer activity in HL-60 cells<sup>53</sup>. These phenolics inhibit arachidonic acid metabolism that has been related to induction of programmed death in certain types of tumour cells<sup>54-57</sup>. This may be due to pro-oxidant activity of these compounds which have been shown to activate transcription factor activator protein-1 (AP-1) in immortalized mouse fibroblast cells in culture<sup>58</sup>. The principal pungent ingredient present in hot red pepper (*Capsicum annum* L.) and chilli pepper (*C. Frutescence* L.) is the phenolic substance named capsaicin (trans-8-methyl-N-vanillyl-6-nonamide) and this substance has been found to induce apoptosis in HL-60 cells in culture<sup>59</sup>. Capsaicin-induced apoptosis has been suggested to be regulated by *bcl-2* and the protein phosphatase calcineurin<sup>59,60</sup>.

Ginger (*Zingiber officinalis* Roscoe, Zingiberaceae) is widely used as a dietary condiment throughout the world. Besides its extensive utilization as a spice, the rhizome of ginger has been used in traditional Oriental medicine. Its major pungent principal is [6]-gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone) and contains minor pungent vanilloids, including [6]-paradol. [6]-Gingerol is

also present in the seeds of Grains of Paradise (*Abramo-mum melegueta* Roscoe, Zingiberaceae) as a major pungent principal<sup>61–63</sup>. [6]-Gingerol and [6]-paradol share common structural features found in chemopreventive diarylheptanoid compounds such as curcumin and yakuchinones. All these phytochemicals have the vanillyl (4-hydroxy-3-methoxyphenyl) moiety and a ketone functional group in their structures. Therefore, the vanillyl ketone functionality may represent an important structural determinant of chemopreventive action by certain categories of phytochemicals<sup>57</sup>. These two compounds show cytotoxic activity through apoptosis<sup>57</sup>. In addition, [6]-gingerol exhibits diverse pharmacological activities, including inhibition of COX and lipoxygenase activities<sup>64–66</sup>. Inhibition of COX could provide a mechanism for induction of apoptosis<sup>48</sup>. Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a phytoalexin present in grapes and other food products, induces apoptosis in both solid and non-solid cancer cells by triggering the CD95 signalling system<sup>67</sup>. It also decreases *bcl-2* expression in HL-60 cells and gives rise to proteolytic cleavage of caspase substrate PARP<sup>68</sup>. Huang *et al.*<sup>69</sup> reported that resveratrol suppressed tumour promoter-induced cell transformation and markedly induced apoptosis, transactivation of p53 activity and expression of p53 protein in the same cell line and at the same doses. Also, resveratrol-induced apoptosis occurs only in cells expressing wild-type p53 but not in p53-deficient cells, while there is no difference in apoptosis induction between normal lymphoblasts and sphingomyelinase-deficient cell lines<sup>69</sup>. In the authors' laboratory experiments conducted in K-562 and HL-60 cell lines curcumin, yakuchinone B and resveratrol exhibited reduced viability through induction of apoptosis (Roy *et al.*, unpublished observations) (Box 1).

## Flavonoids

In past etiological studies, intake of certain kinds of polyhydroxyphenols such as flavonoids or lignans in the diet has been correlated with low incidence of colon cancer and breast cancer<sup>70–72</sup>. A flow cytometric analysis<sup>73</sup> suggested that genistein (a hydroxyisoflavone) induced apoptosis in human pro-myelocytic HL-60 leukaemic cells. Genistein is also reported to inhibit tyrosine kinase<sup>74</sup>, angiogenesis<sup>75</sup> and cell-cycle progression<sup>76</sup>. The flavonoid inhibited prostate cancer (PCa, LNCaP, DU-145, PC-3) cells and H-460 non-small lung cancer cells grown in culture in a dose-dependent manner, which is accompanied by a G2/M cell-cycle arrest. Cell-growth inhibition was observed with concomitant down-regulation of cyclin B, up-regulation of the p21 WAF1 growth-inhibitory protein and induction of apoptosis<sup>77–79</sup>. Zhou *et al.*<sup>80</sup> reported that pure soy isoflavones (genistein, genistin, daidzein and biochanin A) and soy phytochemical concentrate exhibited dose-dependent growth inhibition of murine (MB-49 and MBT-2) and human (HT-1376, UM-UC-3, RT-4, J-82 and TCCSUP) bladder cancer cell lines and also showed the apoptotic feature of cell death. Biflavones isolated from *Selaginella* species exerted tumouricidal effect against various human cancer cell lines<sup>81,82</sup>.

Tangeretin occurring in tangerine peel<sup>83</sup> is extensively used in Japan in Kampo medicines for treatment of cancer patients. Kampo medicines contain some polymethoxyflavonoids, including tangeretin in 100–200 µg g<sup>-1</sup> of dried extracts of mixed herbs containing tangerine peel. Inhibition of membrane sodium pump<sup>84</sup>, extensive single-strand DNA break<sup>85</sup>, inhibition of tyrosine kinase<sup>74</sup> and block of cell-cycle progression<sup>76</sup> have been considered as possibilities for the action of flavonoid on cancer cells. Tangeretin

### Box 1. Apoptosis induced by natural phenolic compounds

Phenolics	Tumour cell lines	Parameters
<i>Phenolic acids and phenols</i>		
Gallic acid, caffeic acid, tannic acid, curcumin, capsaicin, resveratrol, yakuchinones, [6]-gingerol, [6]-paradol	Myelogenous leukaemia (HL-60, ML-1, U-937, THP-1)	Cytotoxicity, DNA fragmentation, morphological characteristics
<i>Polyphenolic flavonoids</i>		
Genistein, genistin, daidzein, biochanin A, tangeretin, baicalein, quercetin, myricetin, apigenin, kaempferol	Promyelocytic leukaemia (HL-60), prostate cancer (PCa, LNCaP, DU-145, PC-3), lung cancer (H-460), bladder cancer (HT-1376, UM-UC-3, RT-4, J-82, TCCSUP), hepatocellular carcinoma (HCC), colon cancer (Caco-2, HT-29)	Cytotoxicity, cell-cycle arrest, morphological characteristics
<i>Tea polyphenols</i>		
(-)-Epigallocatechin gallate, (-)-Epigallocatechin gallate, (-)-Epigallocatechin, (-)-Epicatechin	Lung cancer (PC-9), lymphoid leukaemia (MOLT-4B), stomach cancer (KATO III)	Cytotoxicity, DNA synthesis, DNA fragmentation

inhibited leukaemic HL-60 cell growth partially through induction of apoptosis, while it displayed less cytotoxicity on normal human lymphocytes. Activation of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -dependent endonuclease and *de novo* protein synthesis are thought to be necessary events in the process of tangeretin-induced apoptosis<sup>13</sup>. Matsuzaki *et al.*<sup>86</sup> observed that baicalein, a flavonoid contained in the herbal medicine 'sho-saiko-to', inhibited the activity of topoisomerase II and induced apoptosis in cell lines of human hepato-cellular carcinoma (HCC). Inhibition of topoisomerase II is not by itself sufficient for induction of apoptosis, there may be other mechanisms which can account for the susceptibility of cells to apoptosis induced by baicalein. Dietary flavonoids are polyphenolic compounds known to be antiproliferative and may play an important role in cancer chemoprevention, especially cancers of the GI tract, because of its direct contact with food. Two most potent dietary flavonoids, quercetin and genistein, induced apoptosis in colon cancer cells Caco-2 and HT-29 and in rat non-transformed intestinal crypt cells IEC-6, in a dose-dependent manner<sup>87</sup>.

Flavonoids varied significantly in their antiproliferative potency depending on the structural features. Kuntz *et al.*<sup>88</sup> found that flavonoids baicalein and myricetin were able to induce apoptosis in HT-29 and Caco-2 cells. Flavonoids of the flavone, flavonol, flavanone and isoflavone classes possess antiproliferative effects in different cancer cell lines<sup>88</sup>. The capability of flavonoids for growth inhibition and induction of apoptosis cannot be predicted on the basis of their chemical composition and structure. On the other hand, the structurally related flavonoids such as apigenin, quercetin, myricetin and kaempferol were able to induce apoptosis in human leukaemia HL-60 cells<sup>89</sup>. Experiments conducted with these flavonoids suggest that flavonoid-induced apoptosis is stimulated by the release of cytochrome-*c* to the cytosol, by procaspase-9 processing, and through a caspase-3-dependent mechanism<sup>89</sup>. Furthermore, the potency of flavonoids for inducing apoptosis may be dependent on the number of hydroxyl groups in the 2-phenyl ring and on the absence of the 3-hydroxyl group. This provides new information on the structure-activity relationship of flavonoids.

### Tea polyphenols

Green tea contains several polyphenols, including (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC). Their composition varies depending on the location of cultivation of the tea plant (*Camellia sinensis*), variety of the plant, season of harvest and manufacturing process. EGCG is the main constituent of green tea. Tea polyphenols inhibit growth of human lung cancer cell line PC-9, with the order of potency being EGCG = ECG > EGC > EC<sup>90</sup>. Green tea polyphenols stimulate apoptosis of various other cancer cell lines such as prostate, lym-

phoma, colon and lung<sup>91,92</sup>. EGCG and ECG strongly induced DNA fragmentation in PC-9 cells, the effect of EGC being much weaker than EGCG and ECG. EGCG commonly inhibited TNF- $\alpha$  gene expression and its release from BALB/c-3T3 cells. Reduction of TNF- $\alpha$  level is a key criterion for cancer-preventive agents and this may be the way EGCG induces apoptosis<sup>93,94</sup>. Exposure of human lymphoid leukaemia MOLT-4B cells to extract of persimmon as well as EGC and EGCG led to both growth inhibition and induction of apoptosis in concentration- and time-dependent manner. This result provides the first evidence that catechin compounds derived from sources other than green tea induce apoptosis<sup>95</sup>. It was also observed that exposure of human stomach cancer KATO III cells to black tea theaflavin extract, free theaflavin and theaflavin digallate resulted in growth inhibition as well as induction of programmed cell death<sup>96</sup>. Black tea inhibited DNA synthesis and enhanced apoptosis of nonmalignant as well as malignant tumour cells<sup>97</sup> (Box 2).

### Alkaloids

Solamargine, an alkaloid purified from the Chinese herb, *Solanum incanum*, has been observed to induce apoptosis in human hepatocyte (Hep-3B) and normal skin fibroblast cells in culture<sup>98</sup>. In addition, the gene expression of TNF receptor I was up-regulated within 30 min of solamargine treatment. Since TNF receptor I has been involved in apoptosis, its over-expression may be related to the mechanism of cytotoxicity of solamargine<sup>98</sup>. Alkaloid

#### Box 2. Mechanism of apoptosis proposed for some phenolics

Phenolics	Mechanism
Gallic acid	Generation of reactive oxygen species, $\text{Ca}^{2+}$ influx, activation of calmodulin
Curcumin	Inhibition of cyclooxygenase
Yakuchinones	Inhibition of arachidonic acid metabolism, activation of transcription factor AP-1
Capsaicin	Regulation of <i>bcl-2</i> and calcineurin
[6]-Gingerol	Inhibition of cyclooxygenase
Resveratrol	Triggering of CD-95 signalling system, decrease of <i>bcl-2</i> , activation of caspases, transactivation of p53
Flavonoids	Release of cytochrome C to cytosol, processing of procaspase-9 and activation of caspase-3
(-)-Epigallocatechin gallate	Reduction of TNF- $\alpha$ level

sinococuline, isolated from *Stephania sutchuenensis*, has been found effective in inducing cell-growth inhibition in mouse fibroblast cell line (L-929), HL-60 and a rat alveolar macrophage culture in a dose-dependent manner<sup>99</sup>. Exposure to sinococuline *in vitro* also altered the macrophage function by reducing the production of TNF and reactive nitrogen intermediates. Apoptosis seems to be the mode of death induced by sinococuline<sup>99</sup>. Bioassay-guided fractionation of an extract of a mixture of *Microphilis guyanensis* and *Genipa americana* collected from the rainforest of Suriname yielded the known alkaloid cryptolepine as the major active compound. Several cryptolepine derivatives were synthesized. The structure modification, however, did not result in compounds with a higher potency than the parent compound in the yeast assay system, although some derivatives did show significant cytotoxicity in mammalian cell culture, probably through the molecular mechanism of apoptosis<sup>100</sup>.

Total alkaloids isolated from *Tiliacora racemosa* root containing several bis-benzylisoquinoline alkaloids induced apoptosis in K-562 cells (Taraphdar *et al.*, unpublished observations). Camptothecin (CPT), an alkaloid isolated from the stem wood of the Chinese tree, *Camptotheca acuminata* Decsne, Nyssaceae, is a topoisomerase I inhibitor and induces apoptosis in PLB-985 (a human leukaemia cell line) cells. Sensitivity to CPT was enhanced after neutrophilic differentiation, but was lost after monocytic differentiation. Pyrrolidine dithiocarbamate (an antioxidant inhibitor of NF- $\kappa$ B) and catalase partially inhibited CPT-induced DNA fragmentation in granulocytic differentiated PLB-985 cells. Flow cytometry analysis revealed that reactive oxygen intermediates were generated in CPT-treated PLB-985 cells and suggested that oxygen radicals generated by NADPH oxidase might contribute directly or indirectly to CPT-induced apoptosis in human leukaemia and in neutrophilic differentiated cells<sup>101</sup>. It was reported that 7-hydroxystaurosporine (UCN-01), a natural alkaloid, enhanced *cis*-diamminedichloroplatinum(II) cytotoxicity and apoptosis in ovarian cancer cells<sup>102</sup>. This occurs regardless of p53 status, but wild-type p53 seems to increase the degree of sensitization. Hsueh *et al.*<sup>103</sup> found that UCN-01 enhanced 5-fluorouracil-induced apoptosis and provided a novel way to enhance cellular sensitivity by means of down-regulating E2F-1 protein, a transcriptional activator of thymidylate synthase enzyme. It has also been noted that extended periods of exposure to UCN-01 are needed for optimal manifestation of cytotoxic and apoptotic effects against glioma cells, a factor that must be taken into consideration in the design of future clinical trials with this agent for malignant gliomas<sup>104</sup>. Derivatives of CPT which are topoisomerase I inhibitors and UCN-01, a protein kinase C (PKC) inhibitor, show positive apoptotic response in human breast carcinoma cell lines in culture<sup>105</sup>. MCF-7 and T47D cells exhibited marked resistance to apoptosis, whereas MCF-7/ADR (NCI/ADR-RES) and HS578T cells exhibited the

most pronounced apoptotic response by these compounds. The experiments show that p53 is not required for drug-induced apoptosis. *Bcl-2*, *mcl-1* and caspases 2 and 3 protein levels varied widely, whereas *bax* expression was comparable among cell lines. Interestingly, *bcl-2*, *mcl-1* and *bcl-x(L)* cumulative expressions were inversely correlated with apoptotic response<sup>105</sup>. Another experiment by Sugiyama and his group<sup>106</sup> demonstrated that induction of apoptosis and block of cell cycle in G1 were important determinants of the sensitivity of cancer cells to UCN-01 and suggested that inhibition of CDK 2 activity accompanied by its dephosphorylation and decreased expression level of cyclin A might play an important role in the G1 phase accumulation induced by UCN-01.

### Polysaccharides and glycoproteins

The past decade has seen a dramatic resurgence in research on carbohydrates involved in diseases and their potential use as therapeutics<sup>107</sup>. Several plant polysaccharides have been described with *in vitro* and *in vivo* immunostimulating activity<sup>108–112</sup>. Their major effect seems to be the activation of macrophage cytotoxicity against tumour cells. Likewise, other branched plant heteropolymers have been reported to enhance cytotoxicity of NK cells by inducing the production and/or release of cytokines<sup>113–115</sup>. Some polysaccharides have shown potent activity against various tumours when tested in implanted animals<sup>116</sup>. The mechanism proposed has been the blockage of metastasis by covering galactose-specific binding sites<sup>117</sup>. These activities may have possible therapeutic implications in cancer treatment, from the approach of modulating the immunological functions or by blocking metastasis. A novel cytotoxic proteoglycan, structurally related to arabinogalactan proteins<sup>118</sup>, from the corn of saffron plant (*Crocus sativus* L.) showed a certain level of apoptosis in macrophages in culture with non-cytotoxic concentration resulting in an increase in DNA laddering, characteristic of apoptotic cells. A dose-dependent increase of apoptotic cells was also detected by flow cytometry, confirming this effect. This behaviour was specific for macrophages as no apoptosis was observed in HeLa cells treated with similar proteoglycan concentrations<sup>119</sup>. These results are indicative of apoptosis being a consequence of macrophage activation, as has been suggested previously<sup>120</sup>. Macrophages are implicated in this response by the synthesis of free radicals and oxidative species such as nitric oxide (NO). A wide array of extracellular signals induce macrophage activation and NO synthase expression, increasing the release of NO to the extracellular medium<sup>121</sup>. NO is a reactive molecule involved in multiple responses, including inhibition of tumour cell proliferation. Some membrane-interacting glycoproteins and peptides have shown specific effects in the immune system as consequence of signal transduction pathway activation. Accmannan<sup>122</sup>, cecropin A and mellitin-derived pep-

tides<sup>123</sup> induce macrophage activation, followed by an increase in the free radical NO and peroxides production. The saffron corn proteoglycan induces NO liberation after macrophage treatment with concentrations below toxic limits. Indeed, after treatment with proteoglycan, macrophages increase their apoptotic pattern as occurs after activation with bacterial lipopeptides and lipopolysaccharides<sup>120,124</sup>. Nevertheless, high concentrations of proteoglycan induce membrane damage of macrophages as occurs in tumour cells.

The presence of apoptotic or necrotic effects depending on the dose of proteoglycan has been observed for other membrane-perturbing agents such as cecropin A and melittin-derived peptides<sup>123</sup>. These suggest that the saffron glycoconjugate may be useful in activating macrophages to defend against tumours. It is possible to hypothesize that non-cytotoxic immunostimulating concentrations could be reached in certain tissues when administered *in vivo*. The molecular mechanism that originates macrophage activation by the saffron proteoglycan is mediated through specific stimulation of PKC activity that is probably triggered by the increase in  $\text{Ca}^{2+}$  levels. The rise of intracellular  $\text{Ca}^{2+}$  levels and activation of PKC induce NF- $\kappa$ B-DNA binding, a process necessary to activate iNOS transcription and NO production in macrophages, as has been described for some membrane-interacting peptides such as melittin<sup>123–125</sup>. Compher *et al.*<sup>126</sup> reported that butyrate, produced by colonic bacterial fermentation of dietary polysaccharides such as wheat bran induces apoptosis and decreases proliferation in colon cancer cell lines.

## Lectins

*Viscum album* L. (VAL), a phytopreparation, is being used in adjuvant cancer therapy for several years. Such an extract is reported to stimulate the immune system specifically increasing the number and activity of NK cells and neutrophils<sup>30,32,127</sup>. It also induces production of cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-1 and IL-6 (refs 31–33) and possesses cytostatic and cytotoxic activities on both cultured tumour cells and human lymphocytes<sup>128–132</sup>. This plant contains various toxic proteins, mainly mistletoe lectins (ML) and viscotoxins (VT). The toxic lectins of this plant differ due to their molecular weights and specificities. The MLI shows specificity to D-galactose, whereas MLII and MLIII are specific for N-acetyl-D-galactosamine<sup>133</sup>. All three lectins consist of two chains linked by a disulfide bond. Büssing *et al.*<sup>128</sup> suggested that there might be at least two different ways of cell killing operative in VAL-mediated cytotoxicity: (a) a direct or indirect effect on cell membrane with loss of membrane integrity and subsequent influx of  $\text{Ca}^{2+}$  and phosphatidylinositol, and/or (b) the activation of signalling pathways leading to the typical apoptotic cell death.

There is a receptor-mediated triggering of apoptosis through cross-linking of APO-1/fas and TNF-receptor and a secretory perforin/granzyme-mediated lytic pathway<sup>134</sup>. The granzyme triggers fragmentation of target cell DNA and the perforins induce lysis without induction of DNA fragmentation. Nevertheless, both of them can collaborate to cause typical cytotoxic T cell-induced target cell lysis, including DNA fragmentation. The balance between immune cell activation and apoptosis will be of importance in understanding the protecting and stimulating effects of VAL extracts on the one hand, and killing on the other<sup>135</sup>. VAL extracts showed human lymphocytic cell death and associated changes and several evidences proved that the cytotoxicity was due to apoptosis. Although ML contents of VAL extract strongly correlated with apoptosis-inducing properties, the presence of these proteins will not guarantee its biological activity, indicating the involvement of other components that may modulate ML cytotoxicity<sup>136</sup>. Another study of cytotoxic lectin (KML-C) isolated from an extract of Korean mistletoe [*Viscum album* C.(coloratum)] exhibited typical pattern of apoptotic cell death in tumour cells in culture. Its apoptosis-inducing activity was blocked by addition of  $\text{Zn}^{2+}$ , an inhibitor of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease, in a dose-dependent manner. This suggests that KML-C is a novel lectin whose cytotoxicity against tumour cells is due to apoptosis mediated by  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases<sup>137</sup>.

## Tannins and lignins

Tannins and lignins are present in the natural kingdom in large amounts<sup>138</sup>. Some tannin-related compounds, e.g. tellimagrandin II, remurin B, nobotanin A, nobotanin F, rugosin D, oenothien A, woodfordin D, nobotanin C, woodfordin F and nobotanin K from different plant sources induced cytotoxicity and inter-nucleosomal DNA cleavage in HL-60 cells. Their activity increased with polymerization when their concentration was expressed on a molar basis<sup>45</sup>. Among the lignin-related compounds, natural lignified materials isolated from *Pinus parviflora* Sieb. et Zucc. induced DNA fragmentation whereas alkali lignin and lignin-sulphonate were inactive. A variety of tannins and lignin-related compounds induce DNA fragmentation in different human myelogenous leukaemia cell lines and HL-60 cells<sup>45</sup>.

## Terpenoids

Monoterpenes such as D-limonene and perillyl alcohol (POH) derived from orange peels and lavender, respectively, have been shown to possess chemopreventive properties against mammary, liver and lung carcinogenesis. Reddy *et al.*<sup>12</sup> reported that the colon tumours of ani-

mals fed with POH exhibited increased apoptosis compared to those fed with control diet. Another experiment revealed that apoptosis was induced within 48 h of chemotherapy with POH in advanced rat mammary carcinomas<sup>139</sup>. Gross and ultrastructural morphology of POH-mediated tumour regression indicated that apoptosis accounted for the marked reduction in the epithelial component. D-limonene, the most abundant monocyclic monoterpene in orange peel oil, inhibited the development of gastric cancer through increased apoptosis and decreased DNA synthesis<sup>140</sup>. Limonene attenuates the gastric carcinogenesis enhanced by sodium chloride via increased apoptosis and decreased ornithine decarboxylase activity<sup>141</sup>. Humulone, a bone resorption inhibitor isolated from hop extract, induced apoptosis at low doses in the leukaemia cell line HL-60 (ref. 142). The cytotoxicity of diterpene taxol from *Taxus brevifolia* Nutt. (Tanaceae) represents both inhibition of cell proliferation and cell death. The drug also blocked cells in the G2/M phase of the cell cycle and induced apoptosis<sup>20</sup>. In lower concentration range, taxol stabilized the spindle during mitosis and this mitotic block led to the inhibition of cell proliferation and induction of apoptosis. In higher concentration range, taxol mainly increased the polymerization of microtubule and stimulated the formation of microtubule bundles which blocked entry into S phase and this led to the inhibition of cell proliferation and induction of necrosis<sup>143</sup>. Cell death induced by paclitaxel, on the other hand, occurs via a signalling pathway independent of microtubules and G2/M arrest<sup>144</sup>.

## Isoprenoids

Exogenous isoprenoids farnesol and geranylgeraniol induced actin cytoskeleton disorganization, growth inhibition and apoptosis in A-549 human lung adenocarcinoma cells in culture<sup>145</sup>. One class of phyto-chemicals, broadly defined as pure and mixed isoprenoids, encompasses an estimated 22,000 individual components. A representative mixed isoprenoid, gamma-tocotrienol, suppresses the growth of murine B-16(F10) melanoma cells, and with greater potency the growth of human breast adenocarcinoma (MCF-7) and HL-60 cells<sup>146</sup>. Similar effect has been observed with betalonone, a pure isoprenoid. This compound also suppressed growth of Caco-2 cells<sup>146</sup>. Isoprenoid-mediated suppression of growth is independent of mutated *ras* or *p53* functions. The isoprenoids initiated apoptosis and concomitantly arrested cells in the G1 phase of the cell cycle. The additive and potentially synergistic actions of these isoprenoids in the suppression of tumour cell proliferation and initiation of apoptosis coupled with the mass action of the diverse isoprenoid constituents of plant products may explain, in part, the impact of fruit, vegetable and grain consumption on cancer risk<sup>146</sup>.

## Quinones

Shikonin, a quinone ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL-60 leukaemia cell line<sup>147</sup>. The increase of apoptotic cells by shikonin treatment was preceded by the activation of caspase-3, which plays a central role in apoptotic process. The DNA fragmentation induced by shikonin was completely inhibited by the pre-treatment of z-VAD-fmk, a specific inhibitor of caspase, clearly showing that the mode of cell death was apoptotic.

## Conclusion

Apoptosis or programmed cell death is a series of genetically controlled events that result in the removal of unwanted cells. Apoptosis is an important method of cellular control and any disruption of this process leads to abnormal growth – cancer. Apoptotic cells show a very characteristic morphology as well as specific molecular features. Induction of apoptosis in cancer cells or malignant tissues is recognized as an efficient strategy for cancer chemotherapy. Apoptosis also seems to be a reliable marker for the evaluation of potential agents for cancer prevention. A wide variety of natural compounds appear to possess significant cytotoxic as well as chemopreventive activity. Many of these agents act via apoptosis. Extracts of plants used in traditional medicine also have a similar property. Many more screening studies are necessary using plant extracts and compounds isolated from them. Potential apoptotic inducers should not be cytotoxic to normal tissues and the immune cell system. Naturally occurring compounds that are included in the diet are non-toxic and may partially regulate programmed cell death in several tissues and organs. Elaborate studies with such compounds with respect to their abilities to induce apoptosis and understanding their mechanism of action may provide valuable information for their possible application in cancer therapy and prevention.

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