Agents from amphibians with anticancer properties

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Amphibians have been found to be a source of agents with anticancer properties. Bufalin, for example, is an anticancer agent that may induce apoptosis by its interaction with other genes and cellular components. Certain peptides with anticancer activities have been found in amphibian skin; they include magainins, aureins, citropin 1.1 and gaegurins. These peptides may exert a cytotoxic effect on human cancer cells through various mechanisms. Onconase, amphinase, cSBL (sialic acid-binding lectin purified from Rana catesbeiana eggs) and jSBL (sialic acid-binding lectin purified from Rana japonica eggs), which belong to the RNase A family, were purified from the oocyte cells and eggs of three amphibians, and they induce cytotoxicity by degrading cellular RNA. This paper discusses the medical and pharmaceutical significance of products derived from amphibians. ***Anti-Cancer Drugs*** **19:931–939 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

For centuries, plants have been the major source of active compounds for the development of pharmaceutical products. Plants can either provide an active substance, or provide a product for extraction, purification, or chemical modification to increase the potency of the active substance of interest. Such unmodified compounds are known as 'lead substances'. In recent years, the search for new pharmaceuticals of natural origin has intensified and extended to sources other than plant materials. Amphibians are almost worldwide in their distribution, being found on all the continents except Antarctica [1]. Their range extends from inside the Arctic Circle in the north to as far south as southern Chile and the Patagonian grasslands of southern Argentina. The ability of amphibians to survive in such a broad diversity of habitats may be attributed to the evolution of many different morphological, physiological, biochemical and behavioural adaptations. Amphibians possess a rich arsenal of chemicals that form an integral part of their self-defence system and that also assist with the regulation of dermal physiological action. Currently, a number of agents with anticancer properties have been identified from amphibians.

This paper aims to assess the significance of amphibians as a source of biochemicals, some of which may possess anticancer properties and may thus be of medical interest. We also hope this review will serve as an introduction to a larger field of study and stimulate the interest of a wide range of scientific investigators.

Anticancer agents and secretions from the skin

Amphibian skin is a morphologically, biochemically and physiologically complex organ that fulfils a wide range of functions necessary for the animal's survival. The skin plays a key role in the day-to-day survival of amphibians and in their ability to exploit many widely differing ecological niches. The biochemical elaboration inherent in amphibian skin is necessarily complex given the variety of roles and the range of chemical compounds required for its function. Amphibian skin glands basically consist of only two types – mucus glands and granular glands. The granular glands are the more interesting, as they are the sites of synthesis of a wide range of chemical compounds that provide protection from bacterial and fungal infection, as well as from predators [2,3]. Daly *et al.* [4] divided the compounds found in amphibians into four main categories: biogenic amines, bufodienolides (bufogenines and bufotoxins [5]), alkaloids, peptides and proteins. These broad categories are discussed below, with special attention paid to the molecules that are potentially useful as anticancer agents.

Biogenic amines, bufodienolides and alkaloids

In traditional Chinese medicine, amphibian skin extracts have been used for the alleviation of human suffering [1,6]. Huachansu, a Chinese medicine prepared from dried toad skin, has been widely used in China. Huachansu exhibits significant pharmaceutical activities against certain human cancers, such as hepatoma, gastric cancer, lymphadenoma and oesophageal carcinoma. In India, it was revealed that the skin extract from the Indian toad (*Bufo melanostictus*) is pharmacologically potent, having immunomodulatory and antineoplastic activity on Ehrlich ascite carcinoma-bearing mice, as well as antiproliferative, cytotoxic and apoptotic activities on U937 and K562 cells [7]. Of the three categories,

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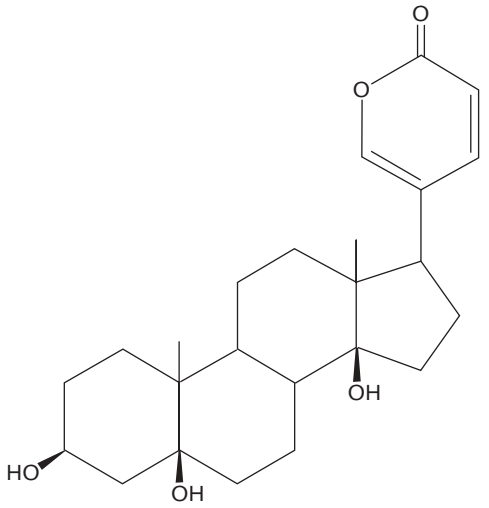
bufodienolides (bufogenines and bufotoxins) may be the most promising for development as anticancer agents. Bufogenines and bufotoxins have cardioacceleratory properties; they can increase the strength of the heart beat and decrease the heart rate. Twenty bufodienolides have been isolated from the traditional Chinese drug 'Chan Su'. All (1–20) of the Chan Su bufodienolides were found to be active against the KB and HL-60 cancer cell lines [8]. Bufalin (Fig. 1) is one of the predominant components of bufodienolides. Bufalin is believed to be in human serum and may be protective against leukaemia. Its pharmacological activities as an anticancer agent have been reported, as shown in Table 1. Zhang *et al.* [25,26] found that bufalin exhibited a potent differentiation-inducing activity in four human myeloid leukaemia cell lines (K562, U937, ML1 and HL60). They also characterized the differentiation of the K562 cell line, and suggested that bufalin in combination with VP16, all-transretinoic acid, lα,25-dihydroxy-vitamin D, rTNF-α or γ-interferon may be very useful in the differentiation of human leukaemia. Yamada *et al.* examined the effect of bufalin on the differentiation of leukaemic cells from acute myeloid leukaemia patients in a primary culture. Bufalin significantly stimulated the functional and morphologic differentiation of leukaemia cells in four of 20 cases, suggesting that bufalin alone is only a modest inducer of differentiation of acute myeloid leukaemia cells in a primary culture. In contrast, acute promyelocytic leukaemia (APL) cells showed synergistic differentiation after treatment with all-transretinoic acid (RA) and bufalin. Therefore, treatment with RA and bufalin may be more effective than treatment with RA alone in the differentiation therapy of APL. A clinical strategy that combines bufalin with a low dose of RA may increase the overall clinical response, decrease the adverse effects of RA and prevent resistance to RA in APL patients [18]. Bufalin induced apoptosis in human tumour cells, such as leukaemia THP-1, leukaemia MOLT-3 and colon COLO320DM cells, but not in normal human peripheral leucocytes and murine leukaemia P388D1, Ml cells and murine tumour cells. Therefore, bufalin or its derivatives might be a useful chemotherapeutic agent for the treatment of human cancers [19]. Bufodienolides in general were found to inhibit growth of human leukaemia cells and were later examined in detail for their growth inhibitory effect on the PLC/PRF/5 liver carcinoma cell line [27]. Bufalin was also found to inhibit endothelial cell proliferation and angiogenesis, and to affect the

Fig. 1



The chemical structure of bufalin.

Table 1 The anticancer activity of bufalin on cell lines and xenograft models

Cell line	Toxicity to cancer cells	Mechanism	Reference
U937	10^{-8}–10^{-7} mol/l	Apoptosis; upregulation of AP-1 through MAPK activity; upregulation of the expression of the Tiam1 gene Transmitted to the nucleus by the translocation of CK2, where it forms a complex with topo IIa and modulates its activity, leading to the induction of apoptosis	[9–12]
HL60, ML1	10^{-7}–10^{-6} mol/l	Apoptosis; downregulation of the expression of the c-myc and bcl-2 genes; causes a marked decrease in the steady state level of topo IIa, which leads to the induction of apoptosis Inhibition of cell proliferation through cell cycle arrest at the G2/M phase	[13–16]
Chinese hamster cells	0.1–0.5 mmol/l	Suppresses topo II and results in a delay in DNA repair	[17]
AML cells THP-1, MOLT-3	1–20 nmol/l	Differentiation-inducing	[18]
COLO320DM cells K562 cells	10^{-6} mol/l	Apoptosis, inhibition of the Na^+, K^+-ATPase and downregulation of the bcl-2 protein	[19,20]
BEL-7402 cells implanted into the liver of nude mice	0.5–1.5 mg/kg	Apoptosis, mediated mainly via the up regulation of bax gene expression	[21]
LNCaP, DU145, PC3	0.1, 0.01 μmol/l 0.01 μmol/l 0.1, 1, 1 μmol/l	Inhibits the proliferation of cancer cells with an elevation of intracellular Ca^{2+} Apoptosis induction	[22]
Bovine aortic endothelial (BAE) cells	5 nmol/l	Proliferation inhibition of endothelial cells through arrest at the G2/M phase of the cell cycle	[23]
Human skin squamous cell carcinoma cells (SSCC-1)	10^{-8} mol/l	Growth inhibitory and differentiation-inducing effects on SSCC-1 cells	[24]

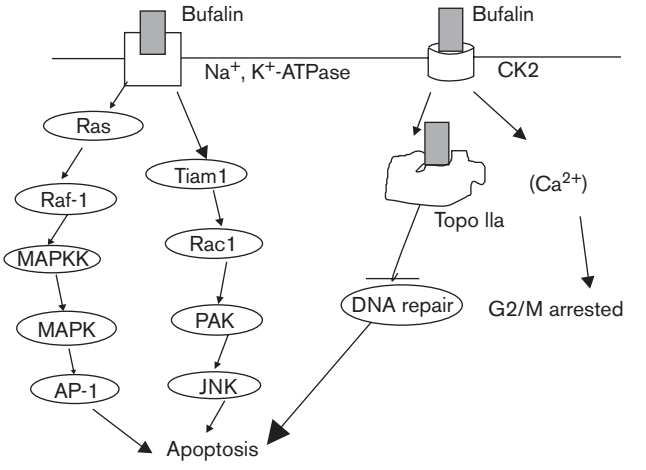
proliferation of prostate cancer cells [28,29]. *In vivo*, bufalin has significant antitumour activities with no marked toxicity in the orthotopic transplantation tumour model of human hepatocellular carcinoma in nude mice, and was able to induce apoptosis of transplanted tumour cells [21].

Apoptosis is an active process that leads to cell death. Apoptosis-inducing agents that are specific for tumour cells might be ideal as antitumour drugs. It has been demonstrated that the expression of certain genes that are involved in oncogenic transformation modulates the induction of apoptosis. In Masuda's study [13], DNA fragmentation caused by bufalin is well correlated with the decrease in the expression of the bcl-2 gene. Downregulation of the cellular myelocytomatosis oncogene was observed at least 2 h before the decrease in bcl-2 expression and DNA fragmentation. These results suggest that bcl-2 may be involved in a downstream step in the cascade of events leading to apoptosis, proximal to the activation of endonuclease, and that DNA fragmentation immediately follows the repression of bcl-2. Bufalin may suppress proliferation by downregulating cellular myelocytomatosis oncogene and may also induce apoptosis by downregulating bcl-2. In Watabe *et al.*'s study, when U937 cells were treated with 10^{-8} mol/l bufalin in the absence of serum, mitogen-activated protein kinase activity was markedly increased 6 h after the start of treatment and remained elevated for 12 h. Together with other findings, it was suggested that the constitutive activation of mitogen-activated protein kinase in response to bufalin in U937 cells is at least one of the signal transduction pathways involved in the induction of apoptosis [9]. Nakaya *et al.* found that when U937 cells were treated with 10^{-7} mol/l bufalin, the expression of both Tiam1 mRNA and protein was induced 1 h after the start of treatment. The increase of Tiam1 mRNA was transient but the level of Tiam1 protein continued to increase for at least 6 h. In addition, the activities of Rac1 and p21-activated kinase were also stimulated by bufalin treatment. These results suggest that the gene expression of Tiam1 was induced by bufalin. Tiam1 might play a critical role in bufalin-induced apoptosis through the activation of Rac1, p21-activated kinase and the c-Jun N-terminal kinase pathway [10]. The most probable candidate as a receptor for bufalin is the Na^+, K^+-ATPase, as the activity of this enzyme in the plasma membranes of various tumour cells is inhibited by bufalin [26]. Treatment for 3 h with bufalin at 10^{-6} mol/l caused a decrease in the plasma membrane potential in several lines of human tumour cells, but not in murine leukaemia cells. No changes in the mitochondrial membrane potential or the release of cytochrome c were observed within 6 h after the start of treatment with bufalin. Moreover, overexpression of bcl-2 in human leukaemia HL60 cells that had been transfected with cDNA for bcl-2 prevented bufalin-induced apoptosis, but did not have a significant effect on the change in plasma membrane potential induced by bufalin. These findings suggest that bufalin induces apoptosis in human tumour cells selectively through the inhibition of the Na^+, K^+-ATPase, which acts upstream of the bcl-2 protein [19]. The specificity of the action of bufalin on human tumour cells might be because of the specific structure of the human Na^+, K^+-ATPase. Further study is needed to characterize the signal transduction pathway that results in the apoptotic action of bufalin. In contrast to earlier reports on the induction of apoptosis in human leukaemia cells, Pastor *et al.* [30] did not observe any DNA damage after protracted treatment with bufalin. They observed that X-irradiated CHO cells pretreated with bufalin showed a dramatic delay in their DNA repair kinetics; it is possible that bufalin influences the repair of X-ray-induced DNA breaks, at least in part, because of its specific effect on topoisomerase (topo) II [17]. This putative mechanism is summarized in Fig. 2.

Peptides and proteins

Amines and peptides are arguably the most important among all the amphibian skin compounds. The discovery of novel peptides, polypeptides and proteins in amphibian granular gland secretions has encouraged the study and exploitation of amphibian biochemicals. Amphibian peptides are of taxonomic significance, and they exhibit antimicrobial properties. After the isolation of magainins from the skin of the African clawed frog *Xenopus laevis*, a number of cationic peptides from the skin of various amphibians were isolated and found to have a broad

Fig. 2



The putative mechanism of apoptosis and proliferation regulated by bufalin. AP-1, activator protein-1; CK2, casein kinase 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; PAK, p21-activated kinase; Rac1, Raf-1: v-raf-1 murine leukemia viral oncogene homolog 1; Ras-related C3 botulinum toxin substrate 1; Tiam1, T lymphoma invasion and metastasis gene 1; TopoIIa, topoisomerases IIa.

spectrum of antimicrobial activity [31]. Recently, it was reported that some peptide derivatives show antitumour activity with little toxicity against nonmalignant cells, either by triggering apoptosis [32–35] or by forming ion channels/pores [36]. Furthermore, some peptides were found to be cytotoxic against MDR cancer cells [37,38]. A well-established example of these peptides is magainin 2, which was originally isolated from the skin of *X. laevis*.

Magainins are a naturally occurring ionophoric class of peptides comprising 21–27 amino acid residues that create an α-helical secondary structure. Magainins are isolated from *X. laevis* skin and they exhibit antibiotic activity against various microorganisms at concentrations having little, if any, toxicity on differentiated erythrocytes [39]. Magainins, especially magainin 2, and their synthetic analogues can rapidly and irreversibly lyse haematopoietic tumour and solid tumour target cells with a relative cytotoxic potency that parallels their antibacterial efficacy, and at concentrations that are relatively nontoxic to well-differentiated cells [36]. *In vitro*, magainin 2 has been shown to exhibit activity against a wide range of cancer cell lines including breast, lung and bladder cancers, as well as melanomas, lymphomas and leukaemias [40–43]. *In vivo*, local treatment with magainin 2 was found to completely ablate a subcutaneous xenograft model of melanoma tumour growth in nude mice. When the melanoma-bearing mice were injected intratumourally with the magainin analogue MSI 511 (D analogue), the tumour completely disappeared in six out of nine mice [41]. Magainin and its D and L analogues were tested *in vivo* on murine ovarian teratoma and leukaemia sarcoma [42] (see Table 2). The D and L analogues were 20–30 times more potent than magainin 2.

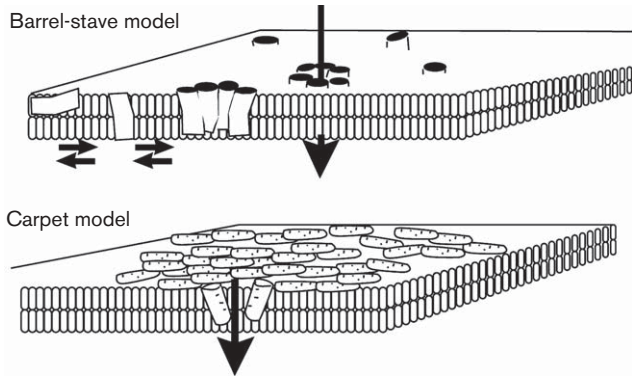
There is some evidence that magainins lyse tumour cells by forming ion-conducting α-helical channels in the cancer cell membrane [36], or via an energy and receptor-independent mechanism to permeabilize and cross the cell membrane [47]. Magainins may also gain access to the cytosolic compartment of cancer cells to trigger the mitochondrial pathway of apoptosis [48]. At present, it is not clear whether magainins kill human cancer cells primarily through membrane lysis and/or apoptosis. A large number of recent biophysical studies have focused on the mechanism of membrane disruption by magainins on model membranes. Five models have been proposed to describe the lytic peptide–membrane interactions. These include the barrel-stave or helical bundle model, the carpet model, the toroidal or two-state model, the detergent-like effect model and the in-plane diffusion model (for a review see [49]). According to the barrel-stave model, magainin at high concentrations inserts into the membranes, while the carpet model is based on observations made at low magainin concentrations [50] (Fig. 3). The basis for selective killing of tumour cells by magainin 2 and magainin analogues has not been completely explained. It is possible that the enhanced expression of phosphatidylserine, a negatively charged species, on tumour cell surfaces creates a target for cationic peptides.

A number of synthetic magainin analogues (magainins A, B and G; MSI-136, MSI-238 and MSI-511) exhibit superior cytotoxic activity against neoplastic cells in comparison to native magainins [36,41–43]. Magainin 2 and two synthetic magainin analogues have been assessed for their in-vivo anticancer activities. The analogue sequence was designed to enhance the amphiphilic α-helical structure and decrease the susceptibility to proteolytic degradation. The novel peptides proved to have enhanced potency *in vitro* and *in vivo* compared with the parent compound. The all-D-amino acid peptide, MSI-238, proved as effective as doxorubicin for a more advanced stage of ovarian tumour, and this activity may be attributed to its resistance to proteolytic degradation. Synthetic magainins A, B and G, all of which are amidated and relatively resistant to peptidase digestion, were at least nine-fold more potent than natural magainin 2 against 8402 cells. Magainins A and G inhibit the growth of human small cell lung cancer cell lines, including drug-resistant tumour cell variants, with an average IC_{50} of approximately 9 μmol/l [43]. They also enhance the effectiveness of the chemotherapeutic agents cisplatin

Table 2 Toxicities of naturally occurring peptides and their synthetic analogues to tumour cells and nonmalignant cells

Peptide	Sequence	Toxicity to normal cells EC_{50} (μmol/l)	Toxicity to cancer cells EC_{50} (μmol/l)	Reference
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	60	200 μg/ml	[36,42,44]
Magainin A	AIGKFLHAAKKFAKAFVAEIMNS	12–25	6–11	[36,43]
Magainin G	AGIGKFLHAAKKFAKAFVAEIMNS	21–34	4–12	[36,43]
Magainin B	GIGKFLHSAKKFAKAFVAEIMNS	>200 μg/ml	17–41 μg/ml	[36]
MSI 511 (D-peptide)		No activity	25 μg/ml	[41]
MSI130 (L-peptide)		No activity	25 μg/ml	[41]
MSI 136	GIGKFLHSAKKFGKAFVKIIMN		10 μg/ml	[42]
MSI 238	GIGKFLHSAKKFGKAFVKIIMN		6 μg/ml	[42]
Gaegurin 5	FLGALFKVASKVLPSVKCAITKKC		13.7–72.0 (IC_{50})	[45]
A4W-GGN5^{N11}	FLGWLFKVASK	240.5 (IC_{50})	23.5–95.5 (IC_{50})	[45]
Gaegurin 6	FLPLLAGLAANFLPTIICKISYKC	>100 μg/ml	4.9–6.5 μg/ml (IC_{50})	[46]
PTP7	FLGALFKALSKLL	>40 (IC_{50})	5–8 μg/ml (IC_{50})	[46]

Fig. 3



Proposed models for the interaction of membrane disrupting lytic peptidesmagainins with phospholipid membranes (modified from Bechinger [49]). The individual steps in the barrel-stave model include: (a) binding of monomers to the membrane as α-helices, (b) aggregation of α-helices into bundles, (c) insertion of the helices into the membrane and (d) recruitment of additional helices to increase pore size. The individual steps in the carpet model include: (a) binding of monomers to the phospholipid head group, (b) alignment of peptide monomers, so that the hydrophilic surface faces the phospholipids, (c) rotation of the molecules and reorientation to the hydrophobic core of the membrane and (d) disintegration of bilayer structure by disrupting the curvature of the target membrane.

and VP-16, suggesting that these synthetic magainin analogues may be used in combination with conventional anticancer drugs to reduce chemotherapy-induced side effects.

Gaegurins are a class of six related antimicrobial peptides that have been isolated from the skin of the Korean frog, *Rana rugosa* [51]. They have a Rana box at the C-terminal end similar to other antimicrobial peptides from the Rana genus, and can be grouped into two families according to their length and sequence similarities [51]. The gaegurins assume a random-coil conformation in aqueous solution but adopt an amphipathic α-helical structure in membrane environments, thus allowing these antimicrobial peptides to mediate cytolysis by the barrel-stave and/or carpet mechanisms [52]. Some gaegurins, particularly those with no or little haemolytic activity, are being considered as target molecules for the development of new antibiotic or anticancer agents [53,54]. More recent studies have revealed that gaegurin 5 and gaegurin 6 that belong to family II of the gaegurins have selective cytotoxic activity against neoplastic cells [45,46]. Gaegurins 5 and 6 each comprise 24 amino acid residues. Gaegurin 5 and two synthetic peptide analogues are able to selectively kill a range of human tumour cell types, including HCT116 colon and MCF-7 breast carcinoma cells, though showing only minimal haemolytic activity [45]. Gaegurin 5 exhibited moderate anticancer activities, with IC_{50} values ranging from 18.98 to 99.57 μg/ml (13.7–72.0 μmol/l) against various tumour cell lines. In particular, the IC_{50} values of A4W-GGN5N11 against some tumour cell lines, such as A498, HCT116, MCF-7 and SK-MEL-2 were comparable with or lower than those of gaegurin 5 (Table 2). These results suggest that the present antimicrobial peptide analogues are also noteworthy as potential compounds for the therapeutic development of anticancer agents, with proper optimization through chemical modifications [45]. Gaegurin 6 and a synthetic peptide analogue (PTP7) have a similar broad spectrum of cytotoxic activity against human cancer cells with no detectable cytotoxicity against peripheral blood mononuclear cells and minimal haemolytic activity. The N-terminal region of gaegurin 6 is indispensable for anticancer activity, whereas the C-terminal region is removable [46] (Table 2). In addition, gaegurin 6 and PTP7 are active against a multidrug-resistant variant of the MCF-7 breast cancer cell line. Gaegurin 6-mediated and PTP7-mediated cytotoxicity may involve apoptosis as DNA fragmentation was detected in MCF-7 breast cancer cells that were exposed to these peptides. However, their cytotoxic potency did not correlate with their antibacterial activity. For anticancer action, two general mechanisms have been suggested: cell necrosis through plasma membrane disruption, or induction of apoptosis through mitochondrial membrane disruption [55]. In either case, the membrane-interacting ability is regarded as the most critical factor for both the antimicrobial and anticancer activities of the peptides.

Other antibacterial and anticancer peptides have also been identified and isolated from amphibians. This group includes aureins 1–3 [56], citropins 1 [57], dahlein 1.2 [58] and maculatin 2.1 [59]. These peptides are all cationic, possessing at least two basic residues at positions 7 and 8, and a free amine at the N-terminal end. The replacement of both basic residues with Ala results in a lack of observable activity. These peptides have also been tested by the National Cancer Institute (Washington) and have activities (IC_{50}) in the 10^{-5}–10^{-6} mol/l range against all classes of human cancers tested (leukaemia, lung, colon, central nervous system, melanoma, ovarian, renal, prostate and breast cancers) [60–62]. The anticancer activity of some of these peptides is moderate.

Anticancer agents from oocyte cells and eggs

Sakakibara *et al.* [63,64] found proteins derived from eggs of the bullfrog, *Rana catesbeiana* (cSBL, sialic acid-binding lectin purified from *Rana catesbeiana* eggs), and the Japanese frog, *Rana japonica* (jSBL, sialic acid-binding lectin purified from *Rana japonica* eggs), which caused agglutination of tumour cells as well as showed lectin activity towards cells with sialic acid residues. It was demonstrated that these proteins belonged to the RNase family [65–67]. Quite independently, Shogen *et al.* [68] showed that a protein from the northern

leopard frog (*Rana pipiens*) oocyte cells (onconase, P30) had antitumour activity and was toxic to cells. They also determined its amino acid sequence and its structure indicated that this protein is also a member of the RNase A superfamily. Recently, another homologue of ribonuclease A named Amphinase (Amph) was found in the oocytes of *R. pipiens*. Four variants (Amph-1–4) were isolated and sequenced [69] and they are listed in Table 3.

Living cells contain approximately 20 exo-ribonucleases and endo-ribonucleases, which process RNA into mature forms and regulate RNA turnover. On account of their ability to degrade RNA and cause cell death, ribonucleases (RNases) can be considered toxins [70]. Among the frog RNases, the four listed below show antitumour activity.

Onconase

Onconase, an egg protein of the leopard frog (*R. pipiens*), has been studied since its discovery by Darzynkiewicz *et al.* [71]. Onconase shows cytotoxic activity against cancer cells *in vitro* [71–75] and *in vivo* [73], and is the only RNase currently in clinical trials. Phase III of its clinical trial as a drug for the treatment of unresectable malignant mesothelioma is now under way [76,77]. Onconase inhibits cell division at the G1 phase at concentrations as low as 1 μg/ml (see review by Youle and D'Alessio [78]). The most interesting property of onconase, in terms of its therapeutic potential, is that the cytotoxicity of this enzyme is affected synergistically by other reagents, such as tamoxifen, trifluoperazine, lovastatin and retinoic acid [72,75], even in the presence of the mdr1 form of multidrug resistance [79]. Although onconase is a frog protein, it is immunologically tolerated in humans with repeated administration and causes only mild and reversible toxicities, possibly because of its homology with the numerous extracellular pancreatic type RNases.

cSBL and jSBL

These two RNases from *R. catesbeiana* and *R. japonica* egg cells show lectin-binding activity towards sialic acid-rich glycoproteins. The lectin from *R. catesbeiana* eggs (cSBL) preferentially agglutinated a large variety of tumour cells but not several types of normal cells. Chemical and physical analysis of the purified lectin indicates that it is a low molecular weight basic polypeptide with five intrachain disulfide bonds [63]. jSBL, at a concentration of 10 μg/ml, causes obvious cytoagglutination of various transformed and tumour cells, whereas erythrocytes and nontransformed fibroblasts were not agglutinated by the lectin. In addition, in-vivo tumour cells were found to be susceptible to agglutination within the range of 10–20 μg/ml [64]. *In vivo*, both SBLs exhibited significant inhibition of solid tumour growth and ascites accumulation after inoculation of Ehrlich cells and sarcoma 180 cells. The survival time of mice inoculated with cSBL-treated sarcoma 180 cells was prolonged, compared with the untreated controls. Sarcoma 180-bearing mice treated with 1000, 100 and 10 μg of cSBL (corresponding to 402, 40 and 4 μmol/l concentrations) had 100, 75 and 50% survival, respectively, after 45 days, and Mep II-bearing mice given single injections of 10 and 5 mg/kg of cSBL had 50 and 17% survival, respectively, after 45 days. Thus, administration of SBLs prolonged the life spans of tumour-bearing mice [80].

Table 3 Members of the pancreatic ribonuclease family from amphibians with natural cytotoxic activity

Name	Species	Molecular mass[a] (Da)	pI	Cytotoxicity *in vitro* IC_{50} (μmol/l)
Onconase	*Rana pipiens*	11 820	9.70[a]	0.01–60
Lectin (cSBL)	*Rana catesbeiana*	11 000	11.8[b]	0.6–6.0
Lectin (jSBL)	*Rana japonica*	13 500	11.8[b]	1.56–6.25
Amph-1	*Rana pipiens*	13 063	10.16[a]	<0.08
Amph-2	*Rana pipiens*	13 077	10.16[a]	<0.08
Amph-3	*Rana pipiens*	13 058	9.95	<0.08
Amph-4	*Rana pipiens*	12 968	10.10[a]	<0.08

[a]Calculated from the amino acid sequence.
[b]Observed data.

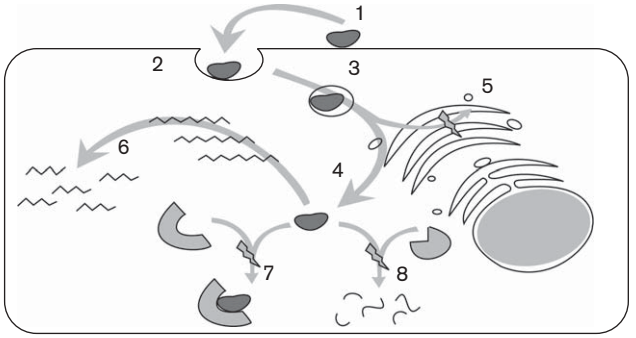
Amphinase

Besides onconase, Shogen *et al.* recently found four variants of another ribonuclease, Amph, from the oocytes of *R. pipiens*. When compared with other well-characterized amphibian ribonucleases, the catalytic efficiency, substrate specificity and glycosylation state of amphinases were found to be novel. Amph variants have cytotoxic activity towards A-253 carcinoma cells which requires intact ribonucleolytic activity when applied at 76.5 nmol/l. Their cytotoxic potencies were similar to that of onconase [69].

The mechanism of the antitumour activity of the amphibian RNases

Chemically modified onconase with *2%* residual activity [81] and genetically modified onconase variants with less than 0.1% residual activity [82] did not prove to be cytotoxic any longer. Therefore, RNases execute cytotoxicity by the degradation of RNA, and with dependence on their catalytic activity. The therapeutic effect of RNases in regard to the selective death of tumour cells is because of the structural and functional differences between normal and tumour cells. Transformed cells are known to contain considerably larger amounts of acid phospholipids and glycoproteins on their surfaces [83]. Therefore, the positively charged RNase molecules will preferentially bind to malignant cells. RC-Rnase (cSBL) and RJ-Rnase (jSBL) cause cell death by the agglutination of cells, which presents sialic acid-containing glycoproteins to their surface [78]. cSBL is selectively internalized by tumour cells having the SBL receptor

Fig. 4



Putative productive and nonproductive routing of RNases as cytotoxins. The RNase molecule (◒) binds to the cell membrane (1) followed by endocytosis (2). Trafficking occurs through endosomes (3) with subsequent release to the cytosol (4) or to nonproductive (◔) organelles (5). In the cytosol the RNases can degrade RNA (〰, 6) leading to cell death or they can be inactivated by ribonuclease inhibitor (✎, 7) or proteases (◒, 8) (From Ulrich Arnold [84]).

through receptor-mediated endocytosis and subsequently induces apoptotic cell death. In contrast to cSBL and jSBL, which cause cell death by binding to the cell surface, onconase has to be internalized to degrade RNA (Fig. 4). Specific protein receptors for onconase have not been found on the surface of either normal or tumour cells, and the RNases are internalized by means of endosomes [85]. Ilinskaya and Makarov [70] have summarized the available data on the possible mechanisms of exogenous RNase transport into cells. They believe that neither receptors nor endocytosis-specific proteins are involved in RNase internalization; their experimental data confirm the involvement of some components specific for tumour cells in RNase-induced cell death [70]. In cell-free assays, cytotoxic amphibian RNases are insensitive to inhibition by a potent inhibitor of pancreatic-type RNases [78]. There is some evidence [86] of a hypothesis that correlates RNase cytotoxicity with RNase inhibitor (RI) insensitivity, but this remains controversial [87]. Some RI-sensitive RNases are selective and potent cytotoxins when internalized by tumour-associated antibodies, and some RNases are tightly inhibited by RI, yet become cytotoxic when successfully routed into the cytosol [88]. Therefore, RI insensitivity does not seem to be a main factor in the antitumour activity of RNases. Up to now, the interactions of exogenous RNases with cellular RNA and cellular components remain unclear in many respects; the role of these interactions may be elucidated in the near future. Deciphering the molecular mechanisms of the cytotoxic effect of RNases will provide a basis for the development of a new generation of antitumour drugs.

In conclusion, amphibians are a source of biochemicals with a variety of anticancer properties, some of which have been identified, and more that will be discovered in the future. Chemotherapy is one of the most important tools for cancer treatment, but complications can arise from the toxic nature of the currently available anticancer agents and from the development of drug resistance. Therefore, molecules derived from amphibian sources might serve as useful alternatives or supplementary treatments for cancer.

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