Amphibian Skin Venoms as a Potential Source of Anticancer Drug Leads

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The pharmaceutical/biotechnology industry is currently focused on the development of biological drugs, often based upon nucleic acids or proteins/peptides – the very molecules that are fundamental to the life process in all organisms and with which all cells are familiar and have been since life began. Monoclonal antibodies (proteins) and small inhibitory RNAs (nucleic acids) are 2 major novel classes of contemporary biological drugs [1,2]. The former have provided some frontline anticancer drugs, such as Avastin. Another source of such biological drugs is animal venoms, which initially seems counter-intuitive, for, after all, these contain toxins that can either debilitate or kill. However, this is exactly what we wish to do to the cancer cells while sparing normal cells – achieving highly-selective targeting while causing minimal collateral damage – something that most current anticancer drug regimes fail to do. Intriguingly, this is exactly the major attribute of many component toxins in venoms, ie to be highly-specific in their targeting. Thus these toxins might be another class of so-called "smart" weapons in the fight against cancer.

Reptile venoms have been the most studied to date and have yielded the lead molecules for ACE inhibitor development for hypertension in the 1960s (bradykinin-potentiating peptides from Brazilian arrowhead viper, Bothrops jararaca, venom) and latterly, for insulin-releasing peptide development for Type-2 diabetes (exendins from the venom of the American Gila monster, Heloderma suspectum [3]. They are not trivial human diseases. Cone shell venoms contain many components that act upon ion channels of cells, some of which are potent analgesics. Scorpion venoms likewise contain a multitude of ion channel toxins and one of them, chlorotoxin, is being assessed for its efficacy in treating brain tumours, notably malignant gliomas. Chlorotoxin binds to a multitude of chloride channels expressed by these tumour cells, but also inhibits the action and expression of the tissue matrix-degrading protease, MMP-2 [3]. However, little focused research for anticancer drug discovery has been carried out on the skin venoms of amphibians.

For most frog and some toad species, skin venoms contain a plethora of peptides that are targeted to molecular binding sites on the cells and tissues of predators. In most instances, they are active on targets in mammals, including human cells. These molecular targets are often specific receptors that normally bind endogenous regulatory factors controlling many aspects of a cell's behaviour. This is why these natural molecules represent a most intriguing source for drug lead-discovery that for the most part remains unexplored. There are ~4,700 known species of frogs and, on average, each produces 100 different peptides. This represents a total of nearly half a million molecules, of which probably < 1% have been structurally and functionally characterised. Of those that are known, anticancer activity has been noted for a considerable number that fall into 5 major categories: cytolysins, protease inhibitors, anti-angiogenics, immune system activators and neuropeptides [4-6].

Cytolysins were originally identified and called antimicrobial peptides (AMPs) due to their broad-spectrum of activity on microorganisms killed by destruction of their cell membranes. Although some were non-selective in their effects, killing mammalian cells as effectively as microbial cells, many were considerably more selective for the latter. The reason(s) for this differential effect is due to differences in membrane composition in microbial (prokaryotic) and mammalian (eukaryotic) cells. The membrane lipid composition of microbes produces a more negatively-charged membrane surface to which the amphibian cytolysins can readily attach. Their lack of membrane-stabilising cholesterol renders them more vulnerable to subsequent destruction. Cancer cells generally have altered membrane lipid compositions compared to normal cells, which involve both an increase in surface negative charge and a reduction in cholesterol. They have greater membrane surface areas than most normal cells due to an increase in the number of microvilli. These factors together render cancer cells a more vulnerable target for the actions of cytolysins thereby providing a higher degree of specificity. Although membrane disruption appears to be the main mechanism by which cytolytic peptides kill cancer cells, there is increasing evidence that other mechanisms are involved. Other targets for the anticancer actions of these peptides include nucleic acids and mitochondria, as well as activation of apoptosis. Although these targets are within the cell, the peptides gain entry through membrane interactions that ensure maintenance of specific targeting of cancer cells. Many analogues of natural amphibian skin cytolytic peptides are being designed with enhanced specificity and potency as anticancer agents [7,8].

Protease inhibitors and their target proteins, the proteases, are ubiquitous in Nature and both play fundamental roles in many life processes. They are of particular importance, as is the balance of their respective actions, in tissue growth, differentiation and repair, all of which are highly-regulated processes. Their deregulation is a characteristic feature of cancer and, not unexpectedly, proteases and their inhibitors are intimately involved. Aberrant protease expression, usually involving up-regulation or more commonly, ectopic expression, is not just a common finding in cancers, but may be central to their aggressive behaviour, such as invasion and metastasis. Matrix metalloproteases (MMPs), are largely responsible for degradation of the matrix proteins of connective tissues that surround cancers, thus permitting growth and directional invasion [9]. In cancers such as malignant gliomas the ectopic expression of MMP-2 facilitates invasion of surrounding neural tissues

leading to poor prognosis. Protease inhibitors could potentially represent a formidable class of anticancer drug and there is indeed increasing evidence to suggest this is the case. The presence of representatives of virtually every major class of protease inhibitor in the venoms of amphibians is most probably related to their regulatory roles in ordered tissue repair, a central aspect of which is the control of cell migration. With this in mind, their selection for this purpose renders them highly-appropriate lead compounds for designing inhibitor drugs for this therapy. A protease inhibitor-enriched preparation of soybeans has anticancer properties and the major proteins belong to a class of protease inhibitor named after its discoverers, Bowman and Birk. Such inhibitors (BBIs) are found solely in nature within the seeds of leguminous plants and grasses [10]. However, BBIs occur widely in amphibian skin venoms and their potencies and target proteases are very similar to their plant counterparts, but they are much smaller in molecular size, permitting deeper tissue penetration [11]. Synthetic replicates of these inhibitors have selective and are potent growth inhibitors of human breast and prostate cancer cell lines. Not all cell lines tested were inhibited, which suggests a highly specific rather than general cytotoxicity, and currently the discrete target through which BBIs mediate this anticancer activity is being sought.

Cancers may arise from single mutated cells and can grow in tissues until their mass approaches several millimetres in diameter, after which needs neovascularisation to acquire to supply the oxygen and nutrients required to sustain growth. Angiogenesis is restricted in adult life to events such as wound repair, vascularisation of the uterine lining during the early menstrual cycle, and pathological events such as macular degeneration and cancer [12]. This illustrates several important points in the understanding of the fight against cancer. There are many common molecular features between normal bodily processes, such as embryogenesis and wound repair, that are appropriate at certain times and under certain circumstances, and those that are hijacked by cancers. Cancer-specific targets for drug development are needed to ensure the minimum of collateral damage to normal body tissues. Angiogenesis is a process that apparently fulfils both of these criteria and is a common feature of the majority of solid cancers. Drugs that interfere with angiogenesis might also be effective in arresting invasion and metastasis. So why have so few drugs been developed for this purpose despite several decades of investigation? Many have been tested, and some can be effective for short periods then fail, while others can be effective in the short term, but were too toxic for continued administration. The former situation is a common feature of anticancer drugs and the latter is a common feature of many natural product and synthetic drugs [13]. Amphibian skin venoms contain many peptide components that have potent anti-angiogenic properties, and these are not used biologically to deter predators, but rather are vital to the regulation of wound healing that is most likely to occur when a frog is attacked. Rapid and regulated skin repair and revascularisation is essential to the repair of the frog skin, a multifunctional organ playing pivotal roles in respiration and excretion. These lower vertebrates may hold the key lead peptides for the design of drugs that will be effective in inhibiting angiogenesis in human cancers. Several peptides of different molecular classes that are effective against several recently identified anti-angiogenic targets are in the process of being clinically evaluated after showing promise in animal models.

The immune system plays a pivotal role in both preventing infection by microorganisms and fighting infections when they take hold. It is difficult to ascertain how many early cancers are contained, at least for some time, by the immune system and any one that finally succumbs could be the exception. The immune system has two components, the innate and the acquired. The innate is the most ancient and shares many molecules found in other organisms. This is a relatively non-specific system in contrast to the

A giant monkey frog (Phyllomedusa bicolor) from the rainforests of South America. This species produces one of the most complex skin secretions/venoms of any amphibian, and is particularly rich in peptides with a vast range of pharmacological effects on human tissues and cells, including some with potent anticancer properties.

acquired response, whose products, the antibodies, rank among the most specifically-targeted proteins occurring in Nature. The skin venoms of amphibians contain many groups of peptides that interact with and activate various cellular elements of the innate immune system. These actions include histamine release from mast cells, induction of leucocyte chemotaxis and potent adjuvant effects in eliciting acquired immune responses [14]. Several classes of these peptides, due to their potent immunostimulatory effects, could have potential applications in the treatment of cancer by either general stimulation of the immune system in cancer patients or by their specific interactions with cancer cells in a manner similar to the cytolysins. One such novel peptide containing 9 amino acids proved to be a most potent cytotoxic agent to a wide range of cancer cell lines in high-throughput screening, but was devoid of activity against microbes. Systematic investigation of this peptide is ongoing.

A major class of bioactive peptides in amphibian skin venoms are neuropeptide analogues. These occur in high diversity, but are often specific for certain amphibian families. These are of considerable interest to molecular bologists as they are structurally and functionally similar to neuropeptides found in mammalian (human) central and peripheral nervous systems. However, often the small structural differences serve to dramatically increase both their potency and stability. Bombesin is one such amphibian skin structural analogue of an endogenous human peptide, and this peptide is a potent mitogen for small cell lung cancer cells. Subsequently, not only did the cancer cells have receptors for the endogenous peptide through which amphibian bombesin acted, but cells themselves produced the endogenous peptide [15,16]. This phenomenon was one of the first examples of autocrine secretion, where cells have growth factor receptors but produce the growth factors themselves. Autocrine stimulation is a well-established phenomenon but is not in itself a good prognostic indicator for cancer cells. Most cancer cells will express specific neuropeptide

receptors, and in most instances their roles in tumourogenesis are not well-established or indeed, understood. A novel tachykinin peptide from amphibian skin that activates its receptors is a potent inhibitor of proliferation of selected human cancer cell lines [17]. This is the first example of a neuropeptide analogue from this source that displays such an activity, and has become the subject of intensive investigation to determine its mechanisms of action.

To summarise, while animal venoms in general are one of the most fruitful resources in terms of drug discovery, there has been little in the way of systematic effort in assessing the value of amphibian skin venoms as anticancer drugs. The small number of species that have been sampled and studied have produced novel molecules of many structural classes and modes of action. It is anticipated that some of these will soon progress to human clinical trials. ■

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