Mechanisms of drug-induced apoptosis in human leukemia
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Chapter 1

General Overview: Apoptosis and Mitogen-activated protein kinases (MAPKs)
1. Programmed Cell Death

At present, cell death is a field that has been attracting much attention leading to several new and important progresses in cell biology. Nevertheless, the recruitment of many new researchers to the field has been leading to some confusion in terms and in the precise classification of the different categories of cell death.

More precisely, cell death has been subdivided into three categories: apoptosis (Type I), autophagic cell death (Type II) and necrosis (Type III). Although the boundaries among these different categories are not always distinct, the different types of cell death may be summarized in the following scheme:

- **Programmed cell death (PCD)** is a natural death of cells during development and/or homeostasis, and it is important for sculpting tissues and destroying harmful cells such as autoreactive immune cells and tumor cells. Excessive PCD may contribute to various degenerative pathologies, whereas lack of PCD can lead to the development of proliferative disorders such as cancer.

- **Apoptosis** is a type of PCD mediated by caspase activation and particular morphological changes. In all works shown in the experimental chapters of this thesis, we will focus on the caspase-dependent apoptosis and use the term apoptosis referring to the classical approach of the caspase-dependent apoptotic way of cell death.
2. Apoptosis

According to the classical approach, apoptosis is a morphologically and biochemically distinct form of eukaryotic cell death that occurs under a variety of physiological and pathological conditions. Apoptotic cells can be recognized by characteristic morphological features: the cell shrinks, shows deformation and loses contact to its neighbouring cells. The chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding and finally the cell is fragmented into compact membrane-enclosed structures which are called "apoptotic bodies" containing cytosol, the condensed chromatin and organelles. The apoptotic bodies are engulfed by macrophages and subsequently removed from the tissue without leading to an inflammatory response. Those morphological characteristics are consequences of biochemical events taking place in the apoptotic cell, such as the activation of proteolytic enzymes that eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm and its organelles. Apoptosis is in contrast to the necrotic mode of cell death in which situation the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue. As consequence, necrotic cell death causes an inflammatory response with cytokine release by the surrounding macrophages.

The cell death with morphological features related to apoptosis may occur in response to various stimuli, such as intracelular stress and cell signalling mediated by specific receptors whereas, any failure on this system of cell death has been associated to a wide range of human diseases such as cancer and the resistance to chemotherapy.

During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing various tissues and organs and allow the organism to get rid of harmful or potentially malignant cells. Apoptosis represents a process of great biological
importance, being involved also in differentiation, development, proliferation, homoeostasis, regulation and function of the immune system. Thus, failures in apoptosis may result in autoimmune diseases, neoplasia and spreading of viral infections. Conversely, excessive apoptosis has been associated with other sorts of diseases, such as AIDS, neurodegenerative disorders and ischaemic diseases.

3. Caspases

The cell death by apoptosis may be executed by external or internal stimuli. The two main pathways of apoptosis induction are the receptor (or extrinsic) pathway and the mitochondrial (or intrinsic) pathway. Both apoptotic signaling pathways converge at the level of the specific proteases: the caspases. The caspases are synthesized in the cell as inactive zymogens called procaspases which at their N-terminus have a prodomain followed by a large and a small subunit. Upon maturation, the procaspases are proteolytically processed, resulting in a small and a large subunit, generating a heterotetramer consisting of each two small and two large subunits, forming the active caspase. There are 14 mammalian caspases identified which usually undergo proteolysis and activation by other caspases in a cascade. Peptide caspase inhibitors can inhibit downstream caspase activation and subsequently apoptosis. The caspases may be grouped into subclasses in various ways and the three different classes of caspases are the following:

- **initiator** caspases that are characterized by long prodomains (more than 90 amino acids) containing either death effector domains (DEDs) or a caspase recruitment domain (CARD);

- **effector** or **executioner** caspases containing short prodomains (caspase-3, caspase-6 and caspase-7);

- the **remaining** caspases which the principal function is related to cytokine maturation rather than apoptosis. Once activated, the prodomains are removed and the large and small subunits are separated by caspase action (all cleavages occur after Asp residues). The active site is formed by the interface
of the two subunits by 1 Arg, 1 His, 1 Cys of the large subunit and 1 Arg of the small subunit. Then, the so activated caspases cleave cellular substrates leading to all fashions of the apoptotic morphology. When activated, the effector caspase-3 cleaves important cellular substrates leading to those morphological features related to apoptosis discussed earlier. Several cellular and viral proteins act as caspase inhibitors. For example, cells contain inhibitor of apoptosis proteins (IAPs) that can inhibit activated caspases. Neuronal cells typically contain such proteins (neuronal apoptosis inhibitory protein, NAIP) to protect them from premature apoptosis. Many viruses also contain viral IAPs, viral anti-apoptotic Bcl-2 proteins or other inhibitors of apoptosis in order to prevent infected cells from dying.

4. The intrinsic (mitochondrial) pathway

Intrinsic apoptosis pathways involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events at the so called apoptosome, a cytosolic death signalling protein complex that is formed upon release of cytochrome c from the mitochondria. In oncology, the intrinsic pathway is activated by DNA damage resulting from cytotoxic chemotherapy as well as irradiation, and also mediates apoptosis from other stimuli including hypoxia, defective cell cycle events, and deprivation of growth factors. Following an apoptotic stimulus, Bax (the prototypic proapoptotic protein), undergoes homodimerization and oligodimerization through interaction of BH3-regions and associates with the mitochondrial membrane with insertion in the mitochondrial membrane, permeabilization resulting in loss of membrane potential, and consequently release of apoptogenic factors including cytochrome c, ATP, and SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pi). ATP binds to the nucleotide-binding domain of apoptotic protease-activating factor 1 (APAF1) resulting in the formation of large oligomers (heptamers). Cytochrome c binds to APAF1 and through a caspase-associated recruitment domain (CARD) in APAF1, binds to a complementary CARD on procaspase 9 leading to activation of caspase 9 and of the downstream “executioner” caspases 3, 6, and 7. These activated caspases
auto-induce activation of themselves as well as downstream caspases and the proteolytic cascade, that ultimately cleaves substrates essential for cell viability, results in the characteristic biochemical and morphological changes of apoptosis.

5. The extrinsic (receptor) pathway

The extrinsic apoptosis pathway is mediated by the activation of death receptors which are at the cell surface and transmit apoptotic signals after binding to specific ligands. The extrinsic apoptosis pathway involves procaspase-8 which is recruited by its DEDs to the death inducing signalling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family. Once bound to the DISC, several procaspase-8 molecules are joined in close proximity and then are capable to activate each other by autoproteolysis. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95 and the TRAIL receptors DR-4 and DR-5. Thereafter, the signaling is transmitted through the cytoplasmic part of the death receptor which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD or TRADD have their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the DISC and the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in apoptosis. Cells that possesses the capacity to induce this direct and mainly caspase-dependent apoptosis pathways are classified as type I cells. On the other hand, in type II cells, the signal coming from the activated receptor does not mediate a caspase signaling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 into the truncated form (t-BID) which translocates to the mitochondria where it acts in concert with
the proapoptotic Bcl-2 family members Bax and Bak to induce the release of 
cytochrome c and other mitochondrial proapoptotic factors into the cytosol. 
Cytosolic cytochrome c is binding to monomeric Apaf-1 which then oligomerizes 
to assemble the apoptosome, the already mentioned complex of wheel-like 
structure with 7-fold symmetry that triggers the activation of the initiator 
procaspase-9. Then, the activated caspase-9 starts a caspase cascade 
involving downstream effector caspases such as caspase-3, caspase-7, and 
caspase-6, eventually leading to apoptosis.

The initial attempts to engage the extrinsic pathway therapeutically 
used systemic and local administration of TNF-a and FAS ligand. However, the 
occurrence of a constellation of adverse clinical events that were consistent with 
a septic shock-like syndrome precluded further development of these agents as 
systemic therapy. Recent drug discovery efforts have focused on the TRAIL 
family of receptors as a target for therapeutic intervention with the goal of 
restoration of normal cellular apoptosis and enhancement of the effectiveness 
of chemotherapeutic agents.

6. Apoptosis regulation

The activation of procaspases is regulated by the Bcl-2 family of 
intracellular proteins. Some members of this family, like Bcl-2 itself or Bcl-XL, 
inhibit apoptosis by preventing the release of cytochrome c from mitochondria. 
Other members of the Bcl-2 family, conversely, are not death inhibitors but 
instead lead to procaspase activation and apoptosis. As an example, BAD 
associates to death-inhibiting members of the family inactivating them, whereas 
others, like Bax and Bak, stimulate the release of cytochrome c from 
mitochondria. If the genes encoding Bax and Bak are both inactivated, cells 
become strongly resistant to most apoptosis-inducing stimuli, indicating the 
pivotal importance of these proteins in apoptosis induction. Bax and Bak are 
themselves activated by other apoptosis-promoting members of the Bcl-2 family 
such as Bid.

In a viable cell, the proapoptotic Bcl-2 family members Bax, Bak, and 
BH3-only proteins are antagonized by antiapoptotic members such as Bcl-2.
response to an apoptotic stimulus, BH3-only members are activated by transcriptional upregulation (Bax, Noxa, Puma), subcellular relocalization (Bim, Bmf), dephosphorylation (Bad) or proteolysis (Bid). Activated BH3-only proteins prevent antiapoptotic Bcl-2 members from inhibiting proapoptotic members. Moreover, they might directly induce a conformational change of Bax and Bak which subsequently oligomerize and insert into the mitochondrial membrane where they form pores either by themselves or by associating with the permeability transition pore complex. In consequence, proapoptotic factors are released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the formation of the apoptosome and the subsequent activation of the caspase cascade.

Another important family of intracellular apoptosis regulators is the IAP (inhibitor of apoptosis) family. These proteins are thought to inhibit apoptosis in two ways: they bind to some procaspases to prevent their activation, and they bind to caspsases to inhibit their activity. IAP proteins were originally discovered as proteins produced by certain insect viruses, which use them to prevent the infected cell from killing itself before the virus has had time to replicate. When mitochondria release cytochrome c to activate Apaf-1, they also release a protein that blocks IAPs, thereby greatly increasing the efficiency of the death activation process. The intracellular cell death program is also regulated by extracellular signals, which can either activate apoptosis or inhibit it. These signal molecules mainly act by regulating the levels or activity of members of the Bcl-2 and IAP families. We see in the next section how these signal molecules help multicellular organisms regulate their cell numbers. Beyond that, the signalling involving the Mitogen-activated Protein Kinases (MAPKs) has an important role in apoptosis regulation as we will discuss in section 8.
7. Programmed cell death type II and III: autophagy and necrosis

Cell death has been subdivided into the categories apoptosis (Type I), autophagic cell death (Type II), and necrosis (Type III). The boundary between Type I and II has never been completely clear and perhaps does not exist due to intrinsic factors among different cell types and the crosstalk among organelles within each type. Apoptosis can begin with autophagy, autophagy can end with apoptosis, and blockage of caspase activity can cause a cell to default to Type II cell death from Type I. Furthermore, autophagy is a normal physiological process active in both homeostasis (organelle turnover) and atrophy. "Autophagic cell death" may be interpreted as the process of autophagy that, unlike other situations, does not terminate before the cell collapses. Since switching among the alternative pathways to death is relatively common, interpretations based on knockouts or inhibitors, and therapies directed at controlling apoptosis must include these considerations.

The autophagic type of death, which is typically seen in large, cytoplasm-rich post-mitotic or only slowly mitotic cells, is characterized by autophagic capture of organelles and particles, substantial expansion of the lysosomal compartment including primary lysosomes, autophagic vacuoles, and secondary lysosomes, and belated collapse of the nucleus. Often organelles appear to be eliminated in waves, for instance one wave in which mitochondria were seen in autophagic vacuoles and afterwards nearly eliminated, and another in which ribosomes or glycogen particles were the primary occupants of autophagic vacuoles. In some instances the lysosomes reside in attacking phagocytes. In others, cell organelles such as mitochondria are sequestered, with any apoptotic morphology delayed until the cytoplasm was nearly completely destroyed. Thus this cells death appears distinct from apoptosis.

"Necrosis" is today the catch-all term for any deaths that do not fit in the other categories described here. Typically, cells entering necrosis lose control of their ionic balance, imbibe water, and lyse. Intracellular proteins in new ionic milieus, often in the presence of high ionic calcium and acid or other abnormal pH, often precipitate. The lysis releases many intracellular
constituents, attracting Mast cells and provoking an inflammatory response. Consequently, the morphology of necrosis is variable and poorly defined.

8. Mitogen-activated protein kinases

Protein kinases and other messenger systems form highly interactive networks to achieve the integrated function of cells in an organism.

To understand the signaling mechanism for any agent, its repertoire of signal transducers and their interactions within this network must be defined in the cellular context. This includes the production of second messengers, activation of protein kinases and the subcellular distribution of these transducers to bring them into contact with appropriate targets. In this context, mitogen-activated protein kinases (MAPKs) exert an essential and multifunctional action.

MAPKs respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. Extracellular stimuli lead to activation of a MAPK via a signaling cascade composed of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). A MAPKKK that is activated by extracellular stimuli phosphorylates a MAPKK on its serine and threonine residues, and then this MAPKK activates a MAPK through phosphorylation on its serine and tyrosine residues. This MAPK signaling cascade has been evolutionarily well-conserved from yeast to mammals. This cascade not only conveys information to the target effectors but also coordinate incoming information from parallel signaling pathways. Such mechanism allow for signal amplification and the generation of a threshold and are subject to multiple activation cascades.

To date, three distinct groups of MAPKs have been characterized in mammals: the extracellular regulated kinase (ERK or p42/p44 MAPK), c-Jun N-terminal kinase (JNK or SAPK1), and p38 MAPK.

In general, ERK1 and ERK2 are key transducers of proliferation/differentiation signals and are often activated by growth factors and phorbol ester (a tumor promoter). In contrast, SAPKs/JNKs and p38 are poorly activated by mitogens but strongly activated by cellular stress inducers such as cytokines,
ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis.

The interactions between MAPK and its immediate upstream kinase (MAPKK) are highly specific: for instance, p42/p44 MAPKs are selectively activated by MEK1 and MEK2, p38 MAPK is selectively activated by M KK3 and M KK6, and JNK is specifically activated by M KK7 and M KK4 under physiological conditions; however, when overexpressed, M KK4 can also activate p38 MAPK.

9. MAPK regulation

Phosphorylation/dephosphorylation - MAPKs are regulated by phosphorylation cascades. In all currently known MAPK cascades, the kinase immediately upstream of MAPK is dual specificity enzyme that can phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues in their MAPK substrate. The duration and amplitude of MAPK activation represents the balance between the activating signal and inactivation mechanisms. The removal of one or both of these phosphates by tyrosine, serine/threonine, or dual-specificity phosphatases dramatically decreases MAPK activity.

Protein complex - scaffolding proteins can bind and organize multiple signaling proteins in a complex by non-catalytic protein–protein interactions; the non-catalytic docking interactions involve protein recognition modules for organization of MAPKs in signaling complexes.

10. MAPKs and cancer therapy

Antitumor agents, despite having diverse primary mechanisms of action, mediate their effects by inducing apoptosis in tumor cells. Cellular commitment to apoptosis, or the ability to evade apoptosis in response to damage, involves the integration of a complex network of survival and death pathways.

Among the best-characterized pathways regulating cell survival and cell death are those mediated by the MAPK family. Not surprisingly, MAPK signaling
pathways have been implicated in the response of tumor cells to chemotherapeutic drugs. While the activities of the major MAPK subgroups are subject to modulation upon exposure of different types of cancer cell lines to diverse classes of antitumor agents, the response tend to be context-dependent, and can differ depending on the system and conditions. Despite these complexities, some important trends have surfaced, and molecular connections between MAPK signaling pathways and the apoptotic regulatory machinery are beginning to emerge. With increased evidence supporting a role for MAPK signaling in antitumor drug action, MAPK modulators may have potential as chemotherapeutic drugs themselves or as chemosensitizing agents. The ability of MAPK/ERK kinase (MEK) inhibitors to block survival signaling in specific contexts and promote drug cytotoxicity represents an example, and recent knowledge of the pro-apoptotic functions of JNK and p38 suggests possible new approaches to targeted therapy.

11. Leukemia

Adult myelopoiesis is a very well regulated cell system with remarkable cellular turnover that constantly regenerates from very few hematopoietic stem cells with self-renewal capacity through a process of cell division and differentiation. While the process is driven by early and lineage-specific growth factors and their receptors, the decisions of differentiation are governed by a set of early acting and lineage-specific transcription factors that regulate the expression of lineage-specific genes. This cell system sets the stage for the pathogenesis of acute myeloid leukemia (AML).

AML is characterized by the clonal growth of immature progenitor cells. Increased proliferation and apoptosis resistance, as well as the inhibition of differentiation are in the center of this pathogenetic event, and it seemed very likely a priori that constitutive and/or aberrant activation of growth factor receptor signaling pathways should contribute. Indeed, both aberrant and constitutive activation of signal transduction molecules have been found in about 50% of primary AML bone marrow samples. The most common of these activating events were observed in the RTK Flt3, N-Ras and K-Ras, and
sporadically in other RTKs. The nature is an inexhaustible source of natural compounds with therapeutic properties for different diseases including cancer. These compounds have shown several interesting effects in animal models and \textit{in vitro} systems.

Studies to date have demonstrated that natural compounds can have complementary and overlapping mechanisms of action, including antioxidant activity and scavenging free radicals, regulation of gene expression in cell proliferation, cell differentiation, oncogenes and tumor suppressor genes, induction of cell cycle arrest and apoptosis.

In this context, violacein and tetrahydroxiquinone present a promissory potential as a coadjuvant of leukemia treatment. The purple-coloured pigment violacein $\left[3\cdot(1,2\text{-dihydro}-5\cdot(5\text{-hydroxy}-1\text{-H-indol}-3\text{-yl})\cdot2\text{-oxo}-3\text{H-pyrrol}-3\text{-ilydene})\cdot1,3\text{-dihydro}-2\text{H-indol}-2\text{-one}\right]$, produced mainly by bacteria of the genus \textit{Chromobacterium}, has attracted increased interest due to its important biological activities and pharmacological potential. The biosynthesis of this indole derivative has been extensively studied and interesting reviews illustrating its production and industrial perspectives, as well as the biological interest in violacein from \textit{Chromobacterium violaceum} have been published

12. Aim of this thesis

\textit{Societal significance in a broader context}

Among the most important relative competitive advantages for a country like Brazil is the availability of the enormous Amazon rain forest with its vast and largely untapped resources. At present the Amazon is mainly used for poorly-sustainable low-technology economic uses, whereas exploiting the jungle for possible medicinal uses is often done by multi national companies offering little benefit for the country itself. I feel that it may be important to exploit the Amazon for the natural products with possible medicinal or other use and build a local knowledge-based industry for such development. If successful, such an industry may also become an important factor in Brazil in the efforts to protect
the forest from lodging. In this thesis I shall embark investigating a few compounds from the Amazonia, focusing on unravelling the molecular mode of action, for their action in leukaemia.

Scope limitation

As may have become clear from this chapter, it is my feeling that successful treatment of leukemia may come from induction of differentiation and apoptosis of the malignant cells, and targeting MAP kinases and survival pathways may be the way forward. To prove this point, I shall address the molecular mode of action for three important compounds, tetrahydroxyquinone, violacein and riboflavin. In chapter 2, the effect of tetrahydroxyquinone (THQ) was investigated in HL60 cells. THQ caused substantial cytotoxicity that coincided with HL60 cell apoptosis through the mitochondrial pathway and was followed by reduced activity of various anti-apoptotic survival molecules, including the protein kinase B pathway. Importantly, transfection of protein kinase B into HL60 cells leading to an artificial increase in protein kinase B activity inhibited ROS-dependent cytotoxicity. More generally, this chapter shows that specific interference of signalling pathways for clinically potentially beneficial effects is possible. Nevertheless, from this work also the limitations of the candidate approach became evident, prompting the development of approaches in which no a priori assumptions as to the biochemical mechanisms mediating pharmacon action are made. To this end in chapter 3, a truly novel approach was taken. Peptide arrays were used to generate comprehensive descriptions of cellular metabolism. The results unambiguously identified the MAP kinase pathway as a major target for violacein, the anti-leukemic purple-colored pigment produced by Chromobacterium violaceum from the Amazon River that stimulates HL60 cells to differentiate into monocytes and granulocytes. That natural compounds may also employ other mechanisms becomes clear in chapter 4, where it is shown that irradiated riboflavin-dependent cytotoxicity is the result of a Fas and FasL-dependent activation of caspase 8. Remarkably, like seen in chapter 2 for THQ, the activation of this
cascade led to an inhibition of survival mediators (PKB and IAP1), as well as downregulation of cell cycle progression regulators. Taken together, these results provide a molecular approach characterizing the riboflavin-mediated apoptosis after photodynamic therapy.

**Conclusion**

Together, this work shows that natural compounds as derived from the Amazonia forest have potential in anti-leukaemia therapy and that it is possible to deduce their mechanism of action. It is likely that such compounds are also useful for other diseases. Thus, the Amazonia jungle may prove a big asset to Brazil in this respect.
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