Screening of necroptosis modulators with potential therapeutic application

Lídia Mafalda Lopes Franco

Supervisor: Prof. Dr. Rui E. Castro
Co-supervisor: Prof. Dr. Cecília M. P. Rodrigues

Dissertation

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The studies presented in this thesis were performed within the Cellular Function and Therapeutic Targeting research group, at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Rui E. Castro, PhD. and Cecilia M. P. Rodrigues, PhD.
Abstract

Programmed cell death is a cellular death process in which a cell is eliminated in response to an inherent and genetically defined set of molecular events. In contrast, non-programmed cell death or classical necrosis is distinguished by its non-physiological or pathological triggering, coupled with lack of requirement for specific internal cellular signaling pathways. For a long time, apoptosis was considered as the sole form of programmed cell death. However, recent evidence suggests that necrosis can also be highly regulated, incorporating features of both necrosis and apoptosis in a process also referred to as necroptosis or programmed necrosis.

The establishment of necroptosis as an alternative form of regulated cell death resulted in the publication of several studies implicating it as the main contributor in the etiology and/or progression of human diseases, such as pancreatitis, ischemic injury and neurodegenerative diseases, among others. Additionally, accumulating evidence indicates that necroptosis is involved in the regulation of cancer. In fact, it is widely accepted that evasion of cell death is one of the hallmarks of cancer. Many lines of clinical and experimental evidence have demonstrated that failure in apoptosis is the most frequent cause of therapeutic resistance; thus, exploring other mechanisms of cancer cell death might lead to the development of strategies to overcome therapeutic resistance.

Ongoing progress in translational research in necroptosis has highlighted the increasing need for the identification of biomarkers of diagnosis, monitoring, and drug development. In addition, only a few small molecules, inhibiting molecular mediators of necroptosis, like Necrostatin-1, have been described. However, and thus far, no inhibitors of necroptosis are available for clinical use.

Targeting regulated necrosis may provide an unprecedented opportunity to develop novel therapeutic strategies. Screening assays of compound libraries will explore biological pathways that can be targeted with small molecules. The subsequent identification of target proteins of newly discovered hits is crucial to understand the cellular mechanism by which the small molecule acts. This project focused on the identification and characterization of
novel modulators of necroptosis, which may ultimately translate into therapeutic strategies for
the treatment of diseases associated with deregulated levels of necroptosis.

In order to identify novel modulators of necroptosis, we performed a biological screening
using a library of twenty-one small molecules and identified three compounds with the ability
to inhibit TNF-α-induced L929 cells necroptosis by more than 80%. To further characterize
the activities of selected compounds, the half maximal effective concentration (EC$_{50}$) for
inhibiting necroptosis and the half maximal inhibitory concentration (IC$_{50}$) for assessing drug
toxicity were determined in the same cell model. Our results show that the selected hits
inhibit necroptosis in L929 cells at micromolar EC$_{50}$ concentrations and were not cytotoxic at
the chosen range of concentrations. In addition, the mechanisms by which the hits inhibit
necroptosis appear to involve, at least in part, inhibition of necroptosis signaling proteins
RIP1 and MLKL.

In parallel, we evaluated the ability of different bile acids, well-established modulators of
apoptosis, in inhibiting necroptosis. Six bile acids and four newly synthesized derivatives
were studied; one molecule inhibited TNF-α-induced necroptosis by more than 60% in L929
cells.

In conclusion, the work presented here describes the discovery of four potential
inhibitors of necroptosis, their confirmation/validation and possible mechanism(s) of action. A
better understanding of the mechanisms of action of these compounds, as well as the study
of their pharmacokinetic properties should help to determine their potential utility in disease
treatment.

**Key words:** bile acids; MLKL; necroptosis; RIP1; screening; small molecules
Resumo

A morte celular programada é um processo através do qual uma célula é eliminada em resposta a um conjunto de eventos moleculares bem definidos. Por outro lado, a morte celular não programada, ou necrose clássica, distingue-se por ser provocada de forma não fisiológica ou patológica, não necessitando de maquinaria celular interna específica. Durante muito tempo, a apoptose foi considerada como a única forma de morte celular programada. Contudo, estudos recentes sugerem que a necrose pode ser altamente regulada, num processo designado por necroptose, a qual incorpora aspetos característicos da necrose e da apoptose.

Após o estabelecimento da necroptose como uma forma alternativa de morte celular, alguns estudos já a evidenciaram como o principal contribuinte na etiologia e/ou progressão de doenças humanas, nomeadamente, em pancreatite, doença isquémica e doenças neurodegenerativas, entre outras. Adicionalmente, a necroptose está envolvida na regulação do cancro. Em particular, a evasão da morte celular é considerada uma das principais características do cancro e diversos estudos clínicos e experimentais têm demonstrado que defeitos na apoptose das células cancerígenas são das causas mais frequentes de resistência à terapêutica. Assim, a investigação de outros mecanismos de morte das células cancerígenas poderá permitir o desenvolvimento de estratégias que visem ultrapassar esta resistência.

O progresso contínuo na investigação da necroptose realçou a necessidade, cada vez maior, de identificar biomarcadores para utilização em diagnóstico, monitorização e desenvolvimento de fármacos. Já foram descritos compostos que inibem elementos da via de sinalização da morte celular por necroptose, como a Necrostatina-1; contudo, ainda não estão disponíveis inibidores da necroptose na prática clínica.

Estratégias que tenham como alvo a necroptose constituem oportunidades únicas para desenvolver novas terapias para o tratamento das doenças anteriormente referidas. Os ensaios de screening de bibliotecas de compostos permitem identificar e, posteriormente, explorar vias biológicas que podem ser atingidas por pequenas moléculas, nomeadamente a
via da necroptose. A subsequente identificação das proteínas-alvo dos compostos bioativos é crucial, uma vez que esta interação é a chave para compreender o mecanismo de ação dos mesmos. Deste modo, este projeto teve como objetivo a identificação e caracterização de novos moduladores da necroptose, os quais podem posteriormente traduzir-se em novas estratégias terapêuticas para o tratamento de patologias que resultem de níveis desregulados de necroptose.

De modo a identificar novos moduladores da necroptose, realizou-se um screening com uma biblioteca de vinte e uma pequenas moléculas e identificaram-se três compostos com capacidade de inibir a necroptose induzida pelo TNF-α em células L929, em mais de 80%. Para caracterizar as atividades dos compostos selecionados, determinaram-se os seus EC<sub>50</sub> (half maximal effective concentration) e IC<sub>50</sub> (half maximal inhibitory concentration) no mesmo modelo celular. Os resultados indicaram que os hits selecionados bloqueiam a necroptose a concentrações na ordem dos micromolar, não apresentando citotoxicidade no intervalo de concentrações utilizado. Além disso, o mecanismo pelo qual os hits inibem a necroptose parece envolver, pelo menos em parte, a inibição das proteínas de sinalização RIP1 e MLKL.

Em paralelo, testou-se a capacidade de diferentes ácidos biliares, moduladores estabelecidos da apoptose, em inibir a via da necroptose. Foram estudados seis ácidos biliares e quatro derivados; um mostrou ser capaz de inibir a necroptose induzida pelo TNF-α em células L929, em mais de 60%.

Em suma, o trabalho apresentado descreve a descoberta de quatro potenciais inibidores da necroptose, a sua confirmação/validação e possível mecanismo de ação. Uma maior compreensão dos mecanismos de ação destes compostos, assim como o estudo das suas propriedades farmacocinéticas irá ajudar a determinar a sua utilidade na clínica.

**Palavras chave:** ácidos biliares; MLKL; necroptose; pequenas moléculas; RIP1; screening,
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<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ALD</td>
<td>alcoholic liver disease</td>
</tr>
<tr>
<td>ASH</td>
<td>alcoholic steatohepatitis</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptotic inducible factor</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocator</td>
</tr>
<tr>
<td>CA</td>
<td>cholic acid</td>
</tr>
<tr>
<td>CDCA</td>
<td>chenodeoxycolic acid</td>
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<tr>
<td>cIAP</td>
<td>cellular inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>cPLA2</td>
<td>cytosolic phospholipase A2</td>
</tr>
<tr>
<td>CYLD</td>
<td>cylindromatosis</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
</tr>
<tr>
<td>DCA</td>
<td>deoxycholic acid</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>Drp1</td>
<td>dynamin-related protein</td>
</tr>
<tr>
<td>EC50</td>
<td>half maximum effect concentrations</td>
</tr>
<tr>
<td>FADD</td>
<td>FAS-associated death domain</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-like inhibitory proteins</td>
</tr>
<tr>
<td>GLUD1</td>
<td>glutamate dehydrogenase 1</td>
</tr>
<tr>
<td>GLUL</td>
<td>glutamate-ammonia ligase</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HIM</td>
<td>homotypic interaction motif</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group box 1</td>
</tr>
<tr>
<td>IC50</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine-pyrrole 2,3-dioxygenase</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>IR</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>L929</td>
<td>murine fibrosarcoma cell line</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LUBAC</td>
<td>linear ubiquitin chain assembly complex</td>
</tr>
<tr>
<td>MLKL</td>
<td>mixed lineage kinase domain-like protein</td>
</tr>
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<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
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<td>NAFLD</td>
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<tr>
<td>NASH</td>
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<tr>
<td>Nec-1</td>
<td>necrostatin-1</td>
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<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<td>NOX</td>
<td>NADPH oxidase</td>
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<td>NOXO1</td>
<td>NADPH oxidase organizer 1</td>
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<td>NSA</td>
<td>necrosulfonamide</td>
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<tr>
<td>OCA</td>
<td>obeticholic acid</td>
</tr>
<tr>
<td>PARP1</td>
<td>poly(ADP-ribose)polymerase 1</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>platelet-derived growth factor receptor β</td>
</tr>
<tr>
<td>PGAM5(L/S)</td>
<td>phosphoglycerate mutase family member 5 (long/short)</td>
</tr>
<tr>
<td>PTPC</td>
<td>permeability-transition pore complex</td>
</tr>
<tr>
<td>PYGL</td>
<td>phosphorylase glycogen liver</td>
</tr>
<tr>
<td>RIP</td>
<td>receptor interacting protein</td>
</tr>
<tr>
<td>RN</td>
<td>regulated necrosis</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
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<td>SMase</td>
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<td>signal transducer and activator of transcription 3</td>
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<td>TAK1 binding protein</td>
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<tr>
<td>TAK1</td>
<td>transforming growth factor-β-activated kinase 1</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<td>tumor necrosis factor receptor 1</td>
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<tr>
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<td>TRAF 2/5</td>
<td>TNF receptor-associated factor 2/5</td>
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<td>TUDCA</td>
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<td>ursodeoxycholic acid</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>zVADfmk</td>
<td>arbobenzoxy-valyl-alanyl-asparty-[O-methyl]-fluoromethylketone</td>
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Introduction and Objectives
Introduction

Programmed cell death plays an important role in regulating the fate of individual cells during development and adult life in multi-cellular organisms [1]. While apoptosis was once considered as one of the most relevant forms of programmed cell death, this view has changed in recent years in light of the discoveries of additional modes of mammalian programmed cell death [2].

Apoptosis is a form of programmed cell death mediated by caspases [3,4]. The execution of apoptosis by caspases leads to distinguishable morphological features, such as exposure of phosphatidylserine (PtdSer) on the outer leaflet of the plasma membrane, chromatin condensation, intranucleosomal DNA cleavage and cytoplasmic membrane blebbing without disrupting its integrity [5,6]. The exposure of PtdSer on apoptotic cells provides a signal for their removal through engulfment by macrophages and other professional phagocytic cells, leading to clearance of dead cell bodies without eliciting inflammatory responses [7]. On the other hand, necrosis is characterized by early plasma membrane permeabilization and organelle swelling. Necrosis frequently occurs when cells are challenged with excessive external stress, such as heat, ischemia, and pathogen infection. The loss of plasma membrane integrity during necrosis leads to leakage of intracellular contents, some of which – such as high mobility group box 1 (HMGB1) – are known as potent pro-inflammatory factors [8]. Necrosis is considered to be accidental and occurs independently of signal transduction pathways. Interestingly, contrary to the traditional belief, recent studies have led to the revelation that regulated forms of necrosis also exist [9]. Among these, necroptosis is probably the most well understood form of regulated necrosis at the moment, at least in molecular terms [10]. In parallel with necroptosis, additional caspase-independent regulated forms of necrosis have been described, for example parthanatos, characterized by overactivation of the DNA damage-responsive enzyme poly(ADP-ribose)polymerase 1 (PARP1), adenosine 5’-triphosphate (ATP) depletion and apoptotic inducible factor (AIF)
nuclear translocation, as well as ferroptosis, which relies on dysfunctional intracellular iron mechanisms.

Compared to the rapidly growing knowledge on the molecular mechanisms that regulate necroptosis, less is known about the relevance of this type of regulated necrosis under physiological conditions or in the pathogenesis and progression of human pathologies. Still, it appears that, defective or excessive cell death by necroptosis can lead to diseases, in a similar way to dysregulation of apoptosis as an underlying mechanism of various pathophysiological states. In cancer for example, there is mounting evidence showing that too little necroptosis can contribute to tumor formation as well as to resistance of established tumors to current cancer therapies.

Necroptosis is increasingly recognized as an emergent clinically relevant therapeutic target. However, important concerns have been raised regarding applicability of the few existing necroptosis modulators. As such, novel specific and potent pharmacological molecules are urgently needed.

1. Molecular mechanisms of necroptosis

Necroptosis is best characterized in the setting of tumor necrosis factor alpha (TNF-α)-induced cell death. In addition to activation by members of the TNF family, necroptosis can also be activated by the Fas ligand, toll-like receptors, lipopolysaccharides (LPS), and genotoxic stress [11,12]. Different kinds of physical-chemical stress stimuli can initiate necroptosis, including anticancer drugs, ionizing radiation, photodynamic therapy, glutamate, calcium overload [13], and virus-mediated activation of DNA dependent activator of IFN-regulatory factors (DAI) [14,15].

The decision on whether death receptor activation results in apoptosis or necroptosis mainly depends on two kinases, receptor interacting protein (RIP) 1 and 3 [1]. RIPs are a group of threonine/serine protein kinases with a relatively conserved kinase domain but distinct non-kinase regions. A number of different domain structures were found in different
RIP family members, and these domains appear to be key factors in determining the specific function of each RIP kinase. RIPs have been demonstrated to play an important role in different biological processes, including those in innate immunity, but also in death-inducing processes [16]. RIP1 and RIP3 interact with each other through the homotypic interaction motif (HIM) at their C terminus region [16]. RIP1 is thought be a crucial kinase making the decision between cell survival or death [14]; its ubiquitination promotes cell survival while de-ubiquitination promotes kinase-dependent cell death [18]. Necrostatin-1 (Nec-1), a small molecule, blocks RIP1 kinase activity and, thus, death receptor-induced necroptosis [19]. Cho et al. reported that RIP3 controls programmed necrosis and augments RIP1 recruitment to the necrosome, a platform allowing engagement of necroptosis [14]. However, in some circumstances, increased RIP3 expression, or the absence of caspase-8, can bypass the requirement for RIP1 in tumor necrosis factor receptor 1 (TNFR1)-induced necroptotic signaling [20,21]. Viral infection and ethanol induced liver injury reflect two physiological circumstances where RIP3 may be activated independently of RIP1.

Under conditions insufficient to trigger apoptosis, TNFα activates TNFR1 which, in turn, recruits RIP1 kinase and other proteins, namely TNF receptor-associated death domain (TRADD), cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, TNF receptor-associated factor 2 (TRAF2) and TRAF5 to form complex I (Fig. 1a). RIP1 is then subject to Lys63-linked polyubiquitylation by cIAP ligases, which allows docking of transforming growth factor-β-activated kinase 1 (TAK1) in complex with TAK1 binding protein 2 (TAB2) or TAB3, as well as of the inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) complex. The assembly of the IKK complex activates the NF-κB pathway, enhanced by recruitment of the linear ubiquitin chain assembly complex (LUBAC) through the linear ubiquitin chains on RIP1. Subsequently, cylindromatosis (CYLD) removes Lys63-linked polyubiquitins (Lys63-Ub) from RIP1, rendering complex I unstable and allowing RIP1 to dissociate from the plasma membrane and interact with TRADD, FAS-associated death domain (FADD), pro-caspase 8 and FLICE-like inhibitory proteins (FLIPs) to form the pro-death complex II (Fig. 1b), which can initiate apoptosis. The long isoform of FLIP (cFLIP_L) and pro-caspase 8 form a
and inactivates RIP1 and RIP3, as well as CYLD, to prevent necroptosis. In the absence of caspase-8 activation, RIP3 can also be recruited to complex II to form complex IIb or the necrosome (Fig. 1c) [22].

**Figure 1:** TNF-induced formation of apoptotic and necroptotic signaling complexes (see text for details). Image adapted from [23].

Increased RIP3 expression is thought to result, at least in part, from increased expression of proinflammatory cytokines and activation of the inflammasome [23]. Mixed lineage kinase domain-like protein (MLKL) represents one of the RIP3 substrates identified so far. RIP3 phosphorylates MLKL at both threonine 357 and serine 358, and these phosphorylation events are required for cell death [25]. Further, MLKL serves as an adaptor to bring the RIP1-RIP3 complex into proximity with other RIP3 substrates. For instance, it increases mitochondrial ROS production by targeting specific mitochondrial proteins [26]. Recent studies have also demonstrated that MLKL is able to translocate to the plasma membrane and trigger cytotoxic influx of either calcium or sodium ions and, hence, disrupt
osmotic homeostasis [27,28]. Nevertheless, a direct pore forming mechanism of MLKL has also been suggested [29,30]. The drug necrosulfonamide (NSA) was identified as an inhibitor of necrotic cell death through a mechanism that prevents MLKL binding to other proteins downstream of the RIP1-RIP3 complex [31]. PGAM5 is one of such proteins and has two splice variants, PGAM5L and PGAM5S [23]. RIP1, RIP3, MLKL and PGAM5L form a dynamic and transient complex that is recruited to PGAM5S on the mitochondrial membrane and then activates dynamin-related protein 1 (Drp1) [32] (Fig 1c, d) inducing reactive oxygen species (ROS) production and membrane permeability, ultimately leading to membrane rupture [23] (Fig 1e). Moreover, the RIP1/RIP3 kinase cascade has also been reported to regulate mitochondrial oxidative stress through c-Jun N-terminal kinase (JNK) [33].

2. Interplay between metabolism, oxidative stress and necroptosis

Among the different stimuli that can induce necroptotic cell death, several metabolism-related molecules and pathways have been identified, including ROS, ischemia-reperfusion injury, calcium overload or ATP depletion [35]. This list of metabolic stimuli points to a connection between metabolism and the necroptosis signal transduction machinery. Metabolic events can regulate the intracellular signal transduction cascade leading to necroptotic cell death, although little is known about the molecular details of this regulation.

Still, accumulating evidence points to redox processes as playing an important role in the regulation of necroptosis. For example, ROS production is rapidly increased during the early stages of necroptosis [36,37], while its inhibition significantly reduces induction of necroptosis [36,38]. These results suggest that ROS are actively involved in mediating necroptosis.

There are several potential intracellular sites that can induce ROS production in the course of necroptosis. Within mitochondria, respiratory chain complexes I and III are considered the main sites for ROS production during programmed cell death [38]. In addition, the mitochondrial adenine nucleotide translocator (ANT) can also contribute to ROS production in the course of cell death. ANT is localized in the inner mitochondrial membrane.
and is responsible for the exchange of ADP against ATP [39]. Inhibition of ANT leads to decreased ADP with concomitant increases in ATP levels in the mitochondrial matrix, which in turn reduce the activity of ATP synthase and induce hyperpolarization of the mitochondrial membrane potential, thereby favoring production of ROS [40]. Because RIP1 has been described to negatively regulate ANT activity, it is tempting to speculate that elevated RIP1 activity during necroptosis may inactivate ANT, thereby favoring the production of ROS. In addition, RIP1 is responsible for phosphorylation-dependent interaction between signal transducer and activator of transcription 3 (STAT3) and the mitochondrial electron transport chain complex I subunit GRIM-19, leading to enhanced mitochondrial respiration and ROS production [41].

Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidases (NOX) constitute another source of ROS [42]. Recently, TNFα has been reported to increase activity of the NOX1 complex via a mechanism involving RIP1 [43]. TNFR1 complex I has also been reported to serve as platform enabling the docking of NOX1 at the plasma membrane, thereby promoting the generation of ROS [43]. This process involves TNFα-dependent association of NADPH oxidase organizer 1 (NOXO1) subunit with RIP1, TNFR-associated death domain protein (TRADD) and riboflavin kinase, which in turn results in ROS production via NOX1 [43]. ROS generation by NOX1 may result not only in lipid peroxidation and membrane damage but also engage a feed-forward amplification loop to trigger further ROS production via the mitochondrial respiratory chain. Another amplification loop may involve the lysosomal compartment, where hydrogen peroxide can interact with ferrous ions to produce hydroxyl radical, a highly reactive ROS species [44]. Such amplification loops can lead to overproduction of ROS at the mitochondrial respiratory chain. This bears the danger of a lethal vicious cycle eventually resulting in the generation of reactive nitrogen species (RNS). RNS species can function as oxidants to produce protein or lipid oxidation, thereby altering protein function and causing membrane damage [45]. Downstream of the RIP1/RIP3 necrosome complex, different metabolic pathways, including glycogenolysis and glutaminolysis, have also been suggested to mediate signaling events during necroptosis.
RIP3 has been shown to promote the activity of several metabolic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1) [37]. PYGL is a key enzyme in the catabolic metabolism of glycogen, mediating the conversion of glycogen into glucose-1-phosphate, which in turn can be modified into glucose-6-phosphate, a substrate for glycolysis that can promote the generation of ROS through a mitochondrial metabolic burst. In addition, RIP3 was shown to activate GLUL and GLUD1 [37], two glutaminolysis enzymes. Enhanced glutaminolysis can engage the Krebs cycle, eventually contributing to increased ROS generation. Thus, RIP3-dependent changes in glycogenolysis and glutaminolysis can result in enhanced energy metabolism and increased ROS production.

Intracellular ATP content constitutes a central regulator in the decision on the mode of cell death. Upon depletion of ATP, human T-cells have been reported to switch from apoptosis towards necrosis. In this model, the generation or addition of ATP results in restored ability of T cells to undergo apoptotic cell death [46]. Apoptosis is an ATP-dependent cell death mechanism and different steps in the apoptotic signaling cascade are dependent on an adequate supply of bioenergetic substrates and ATP consumption. These include the activity of the translational machinery, protein degradation via the ubiquitin proteasome system, and activity of DNA repair enzymes such as Poly (ADP-ribose) polymerase 1 (PARP1) [47-49].

PARP1 has been described to play an important role in the metabolic regulation of cell death. PARP1 is localized in the nucleus and becomes activated upon extensive DNA damage [50,51]. PARP1 overactivation leads to NAD, ATP and an overall acute bioenergetic depletion, which promotes the release of apoptosis-inducing factor (AIF) from the interspace of mitochondria and its translocation to the nuclear compartment [52]. Within the nucleus, AIF is supposed to be required for large-scale DNA fragmentation in a caspase-independent manner [52].

Other molecular mechanisms that contribute to the execution of necroptosis upon assembly of the RIP1/RIP3 necosome are: 1) activation of JNK-mediated degradation of
ferritin, thus increasing the labile iron pool and, consequently, ROS formation and 2) sphingomyelinase (SMase)-mediated generation of ceramide, which is converted into sphingosine by ceramidase and promotes a cytosolic Ca\(^{2+}\) wave that activates calpains and cytosolic phospholipase A2 (cPLA2). cPLA2 triggers lipid peroxidation by mobilizing the lipoxygenase substrate arachidonic acid (AA). AA is converted by lipoxygenase into membrane-damaging lipid hydroperoxides [52]. Finally, sphingosine, calpains and lipid hydroperoxides induce lysosome membrane permeabilization (LMP), resulting in the leakage of cytotoxic hydrolases into the cytosol. The production of ROS from all these sources initiate vicious cycles of damage by exacerbating mitochondrial uncoupling and lipid peroxidation and favoring the opening of the permeability-transition pore complex (PTPC). This results in the permeabilization of mitochondrial membranes and the translocation of cytotoxic proteins, including AIF, from the mitochondrial intermembrane space to the cytosol.

### 3. Necroptosis-associated pathologies

Cell death is a common feature in several human diseases such as ischemia-reperfusion injury and neurodegeneration. Although inhibition of apoptosis in many animal models of human diseases demonstrated the benefits of blocking cell death, the lack of “druggable” targets in the apoptosis pathway has so far prevented the development of apoptosis inhibitors for the treatment of human diseases. The discovery of necroptosis and RIP1 kinase as a “druggable” target in the necrototic pathway elicited significant interest in studying the implication of necroptosis in human diseases, as well as the potential benefits of its inhibition.

#### 3.1. Ischemia-reperfusion (IR) injury

Ischemia is caused by obstruction of blood flow to a tissue, resulting in limited supply of oxygen and nutrients and, if prolonged, in an impairment of energy metabolism and cell death. Restoration of the blood flow (reperfusion) results in oxygen reintroduction, neutrophil infiltration, cytokine production and generation of ROS, leading to cell death associated with
inflammation [44,53]. Interestingly, Nec-1 was shown to reduce inflammation and the infarct size caused by experimental myocardial IR [54-56], and reduced tissue injury upon cerebral [19,57], retinal [100] or renal IR [58,59] and neonatal hypoxia-ischemia in the brain [60,61], by preventing oxidative stress, necrosis and inflammation. Moreover, Nec-1 inhibited RIP3 upregulation, RIP1-RIP3 complex formation and phosphorylation, and MLKL recruitment to RIP1, in neonatal hypoxia-ischemia and myocardial IR [54]. RIP3-deficient mice also show reduced injury upon renal IR [58], as well as decreased cardiac hypertrophy and inflammation after myocardial infarction [62].

Noteworthy, both preocclusion and postocclusion delivery of Nec-1 exerts protective effects [19,56], suggesting that Nec-1 effectively mitigates not only ischemic but also reperfusion injury. An active role for necroptosis has also been demonstrated in animal models of traumatic brain injury [63,65], traumatic spinal cord injury [64], and intracerebral hemorrhage [63], which may share a part of the disease mechanism with IR. In addition, kidney transplantation is inevitably accompanied by IR injury, and RIP3 deficiency reduces injury and improves function of donor kidneys [66].

Taken together, these data indicate that necroptosis is a crucial component of IR injury. However, simultaneous inhibition of both apoptosis and necroptosis had a synergistic effect on cerebral IR injury [57]. Moreover, another study showed that both RIP1/RIP3- and CypD-dependent regulated necrosis participates in renal IR injury by two independent pathways [58]. Different forms of regulated necrosis may thus contribute to IR.

3.2. Neurodegenerative diseases

Huntington’s disease (HD) is an autosomal dominant disease characterized by motor and cognitive deficits, as well as psychiatric symptoms. The pathology is mainly due to the aggregation of mutant polyQ huntingtin protein, leading to neuronal dysfunction and cell death. Nec-1 reduces cell death in an immortalized striatal neuronal line expressing mutant HTT, and delays the onset and progression of disease in the mutant HTT-expressing R6/2
transgenic mouse model [75]. Furthermore, increased expression of RIP1 was observed at the onset of disease symptoms [75]. However, the survival benefit remained modest, indicating that necroptosis is probably not the only cell death mechanism contributing to neuronal cell death in this model.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative condition with loss of motor neurons. Using a coculture system of primary motor neurons with astrocytes from ALS patients, it has been shown that both familial and sporadic ALS astrocytes compromise neuronal survival in a RIP1 kinase-, MLKL-, and Bax-dependent manner, while motor neurons death can be rescued by Nec-1 [67]. Therefore, RIP1 inhibition could potentially benefit both familial and sporadic ALS patients. The search for a common neurotoxic factor in ALS astrocytes is underway, which may shed light on the mechanistics of ALS pathogenesis.

Alzheimer’s disease (AD) is characterized by a progressive loss of memory and motor functions. Histologically, the disease is due to both the formation of extracellular β-amyloid plaques and neuronal cell death in the cerebral cortex and other subcortical regions. It was recently shown that Nec-1 administration decreases aluminum-induced neuronal cell death, inhibits expression of AD-related proteins and improves learning and memory retention in a mouse model of AD [68].

Retinal degeneration accounts for vision loss in the aged population and in patients with genetic disorders such as retinitis pigmentosa. In the rd10 mouse model of retinitis pigmentosa, the expression of RIP1 and RIP3 is augmented. Blockade of RIP1 or deletion of RIP3 preserves the survival and function of cone cells which are majorly responsible for daylight vision [69]. In dsRNA-induced retinal degeneration, a mouse model of age-related macular degeneration, RIP1 and RIP3 are also required for cell death and inflammation of retinal pigment epithelium and photoreceptors, and the degeneration of retinal pigment epithelial cells [70]. Given the sensitivity of retina to necroptosis, RIP kinases might be targeted for therapeutic intervention to treat degenerative vision loss.
3.3. Pancreatitis

Both apoptotic and necrotic cell death mechanisms have been observed during acinar cell death [71]. Of note, the severity of pancreatitis directly correlates with the extent of necrosis. Switching cell death to apoptosis, by pharmacological caspase activation, results in a milder form of pancreatitis, illustrating the harmful nature of in vivo necrosis [71,72]. Importantly, RIP3-deficient mice are protected from cerulein-induced pancreatitis, demonstrated by the absence of pancreatic acinar cell loss and necrosis, as well as lower levels of serum amylase [73]. RIP3 expression has been shown to correlate with the sensitivity of pancreatic acinar cells to undergo necroptosis, as RIP3 expression was dramatically increased in WT mice pancreas, which most probably primes the cells for subsequent necroptosis [73]. Interestingly, Mlkl-deficient mice were also reported to be partially protected from cerulein-induced pancreatitis [74], thus confirming necroptosis involvement in the pathogenesis of this disease. Surprisingly, a recent study showed that Nec-1 administration does not elicit any protective effects in this context [90]. Still, it is possible that these negative results are reflecting issues of Nec-1 pharmacokinetics [76,77].

3.4. Sepsis and systemic inflammatory syndromes

In recent years, several research laboratories have shown that regulated necrosis appears to be involved in sterile inflammation and septic shock. Duprez et al. were the first to demonstrate that targeting RIP1 (by Nec-1) or RIP3 (RIP3 deficiency) can substantially improve survival in a model of TNF-induced systemic inflammatory response syndrome, and in cecal ligation and puncture as a model for sepsis [78]. The authors showed that, although RIP3 deletion was protective in the cecal ligation and puncture model of sepsis, it did not affect the levels of bacteremia itself. This work indicates that RIP1/RIP3-mediated necroptosis most probably acts downstream of bacterial infection in the inflammatory phase of sepsis [79]. Because RIP1-deficient mice die shortly after birth [80], a new strategy to reveal the function of RIP1 kinase activity in vivo was recently developed by generating RIP1
kinase dead knock-in mice. Like RIP3−/− mice, these RIP1 kinase dead knock-in mice are completely protected against a lethal dose of TNF [81]. It was shown that the drop of the body temperature, as a result of TNF-induced systemic inflammatory syndrome, is prevented in MLKL−/− and RIP3−/− mice [82]. Strikingly, MLKL−/−Caspase-8−/− mice and RIP3−/−Caspase-8−/− mice are completely protected against hypothermia during TNF injection [82]. These data suggest that TNF-induced hypothermia is also a consequence of caspase-8-dependent apoptosis and MLKL-dependent necroptosis, although it can also be interpreted that in the absence of caspase-8 the response is completely biased to necroptosis, as blocking caspase-8 sensitizes the necroptosis pathway [83]. This is in line with reports on the sensitization to TNF-dependent necrotic cell death in CrmA-transfected cells [9,84], and on TNF-mediated systemic inflammatory syndrome by combined injection of TNF and zVAD-fmk [85]. Further, caspase-8 prevents RIP3-mediated necroptosis during development [82,86].

Of note, two other studies demonstrated that administration of Nec-1 sensitized the lethal outcome in TNF-induced systemic inflammatory response syndrome and sepsis induced by cecal ligation and puncture [77,78]. Dose-dependent effects of Nec-1 in vivo could explain these contradictory findings. Remarkably, lower concentrations of Nec-1 apparently aggravate TNF-induced systemic inflammatory response syndrome, instead of being less protective [76,77], suggesting that in vivo pharmacodynamics may affect the outcome. The protective role of RIP3 in cecal ligation and puncture is also controversial. A recent article demonstrated that RIP3−/− mice have a higher mortality rate than controls in the cecal ligation and puncture model [74]. These contradictory results are not fully explained, but the outcome of the cecal ligation and puncture model is highly susceptible to experimental variation and animal housing conditions. A recent study showed that LPS-induced sepsis was not affected by catalytically inactive RIP1 or RIP3 deficiency [82], suggesting that RIPKs are not directly implicated in LPS endotoxemia. Of note, recent definitions and concepts of sepsis emphasize the dysregulated host response to illness [87] and, thus, additional studies are required to delineate the precise modulatory role of cell death modalities (i.e. necroptosis) in the host response during sepsis.
3.5. Acute kidney injury (AKI)

AKI is a common clinical problem associated with high mortality, which can occur due to sepsis or kidney IR. Previous studies have shown that both apoptotic and necrotic cell death are involved in the pathogenesis of AKI caused by nephrotoxic injury [88]. Moreover, inhibition of apoptosis in HK-2 human proximal tubule cells treated with cisplatin switches cell death towards necroptosis, which is possible of prevention by Nec-1 [89].

Several in vivo studies have shown that Nec-1 prevents osmotic nephrosis and cisplatin-induced AKI [72,90,91], whereas the pan-caspase inhibitor zVADfmk does not [88]. Moreover, both RIP1/RIP3- and CypD-dependent necrosis participate in renal IR injury, but contribute independently to the pathology [58]. These results suggest that necroptosis may be a dominant factor in the pathogenesis of AKI. In addition, Nec-1 is also able to prevent dilation of peritubular capillaries [88], suggesting a role for RIP1 in the regulation of microvascular hemodynamics and pathophysiology of AKI.

3.6. Pathogen invasion

Programmed cell death is often harnessed by the host to control intracellular growth of infected pathogens. Conversely, it can be critical for viruses to maintain the survival of infected cells in order to support its replication.

3.6.1. Viral infections

Vaccinia virus (VV) encodes the caspase inhibitor B13R/Spi2, which blocks apoptosis upon infection, but sensitizes cells to RIP1/RIP3-dependent necroptosis. Indeed, after VV infection, the pro-necrotic RIP1-RIP3 complex assembles in the liver, concomitantly with induction of TNF expression and inflammation [14]. In contrast, necrotic tissue injury and inflammation are significantly reduced in RIP3-deficient mice [14] or RIP1 D138N/D138N mice [81], resulting in highly elevated viral replication and mortality, as in Tnfr1- and Tnfr2-deficient mice [12,14,92].
Murine cytomegalovirus (MCMV) not only encodes a caspase inhibitor but also a necrotic inhibitor, vIRA, which is required to prevent premature host cell death upon infection [93,94]. vIRA contains a RHIM domain, enabling its interaction with RIP1 and RIP3. Accordingly, vIRA inhibits necrotic cell death in response to TNF by disrupting the pronecrotic RIP1-RIP3 complex and suppressing NF-κB and p38 activation [28,95,96]. Strikingly, upon MCMV infection with a strain carrying a RHIM-mutated vIRA, necrosis can be rescued by RIP3 KO, but not by Nec-1 [28], confirming that RIP3-dependent-, but RIP1-independent- necrosis functions as a potent anti-viral defense mechanism. Finally, a recent study revealed that the DNA sensor DAI interacts with RIP3 through a RHIM domain, mediating MCMV-induced necroptosis [15].

Similarly, MC159, encoded by the poxvirus Mollusucm contagiosum, possesses anti-apoptotic activity [97], and also inhibits RIP1-dependent necroptosis in response to TNF [12]. Interestingly, interaction between RIP1 and MC159 has also been demonstrated [98]. Other viral DED-containing inhibitors, such as equine herpesvirus-2 E8 and human herpesvirus K13, have also been demonstrated to protect cells from death receptor-mediated necrosis, and thus may serve similar roles in establishing efficient viral infection [12].

3.6.2. Bacterial and parasite infections

Despite the above studies highlighting the relevance of necroptosis as part of the immune defense against virus, excessive necroptosis of activated T cells or macrophages in response to infection can also halt the development of an efficient immune response against pathogens. This has been illustrated, for instance, in T cell-specific Fadd-deficient mice, which are more susceptible to Toxoplasma gondii infection than wild-type mice [99], as well as in macrophages infected by Listeria monocytogenes under cIAPs inhibition [100] or infected by Salmonella enterica serovar Typhimurium [101]. These studies pinpoint the role of FADD and cIAPs as crucial necroptosis regulators in immune cells during infections. Finally, a study performed in zebrafish showed that macrophages infected by M. tuberculosis die by RIP1- and RIP3- dependent necroptosis upon TNF treatment [102].
Bacterial infections can also lead to severe hemorrhagic disorders associated with induction of necrosis. For instance, *Clostridium perfringens* type C strain causes fatal hemorrhagic enteritis in several animal species and humans [103]. A recent study showed that *Clostridium perfringens* β-toxin, which is the essential virulence factor of *Clostridium perfringens* type C, induces necrotic cell death of porcine primary endothelial cells characterized by ATP depletion and LDH and HMGB1 release [104]. Cell death is inhibited by Nec-1, indicating that necroptosis appears to be involved in the pathogenesis of hemorrhagic enteritis.

### 3.7. Liver diseases

Only a few studies have evaluated the impact of necroptosis in liver diseases. Hepatocytes are susceptible to TNF-related apoptosis-inducing ligand (TRAIL)-induced necroptosis, which is dependent on formation of the RIP1/RIP3 complex. Indeed, Nec1 protects against concanavalin A (ConA)-induced hepatitis in mice [105,211]. In this regard, Liedtke *et al.* addressed the differential effects of TNF-induced liver injury in hepatocyte-specific caspase-8 knockout mice. These mice are protected from liver failure induced by Fas and TNFR1, but injection of ConA results in marked oxidative damage, spontaneous liver inflammation and enhanced non-apoptotic liver injury [106,107]. Combined deletion of hepatocyte-specific caspase-8 and NF-κB essential modulator (NEMO) further exacerbates liver necrosis and cholestasis [106]. Compared with Fas-induced liver injury, ConA-injury is mediated by membrane-bound TNF and does not require suppression of NF-κB. Further, this process is also caspase-independent, which points to necroptosis as the cause of liver cell death.

Non-alcoholic fatty liver disease (NAFLD) represents the most common chronic liver disease in the Western world [108,109]. It comprises a spectrum of liver lesions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), a more aggressive disease entity within the spectrum, associated with inflammation, cell death, and fibrosis. NASH can
progress to cirrhosis and, ultimately, hepatocellular carcinoma (HCC) [110]. Among all the different cell death pathways, apoptosis is the most well-described and established form in NAFLD [107,111,112]. However, it was recently demonstrated that human NASH livers express high levels of RIP3 [113]. More importantly, recent results have demonstrated that necroptosis is increased in the liver of NAFLD patients and in experimental models of NASH. Further, TNF-α triggers RIP3-dependent oxidative stress during hepatocyte necroptosis, suggesting that targeting necroptosis may arrest or at least impair NAFLD progression [113]. Indeed, despite the current NAFLD burden, no specific and effective pharmacological therapy is available. A pharmacological inhibitor of necroptosis could impact on the progression of NAFLD, preventing fibrosis, cirrhosis and hepatocellular carcinoma.

At the same time, and due to worldwide increase in alcohol abuse, alcoholic liver disease (ALD) has become one of the most common causes of liver-related morbidity and mortality. Alcoholic steatohepatitis (ASH) is the severe form of ALD, and may progress to fibrosis, cirrhosis and HCC [114]. In ALD, there is evidence for both apoptosis and necrosis [115,116]. As such, inhibition of hepatocellular death may also prevent ALD progression to fibrosis and HCC. Direct targeting of apoptosis and/or necroptosis, as well as mechanisms that indirectly prevent cell death, such as the involvement of the gut microbiota-liver axis or ROS, are potential relevant targets.

Hepatocellular carcinoma (HCC), the most common primary liver tumor, arises almost exclusively in the setting of chronic hepatic inflammation [117]. Cell death represents a key trigger of inflammation, thus contributing to multiple hallmark capabilities of cancer [118]. In chronic liver disease, hepatocyte cell death is a prominent feature driving progression to hepatic fibrosis and HCC [119]. One of the challenges in clinical cancer therapy is the resistance of cancers to apoptosis. In HCC, apoptosis resistance is one of the most significant factors for hepatocarcinogenesis and tumor progression, leading to resistance to conventional chemotherapy [120]. Since many anti-cancer drugs are inducers of apoptosis, inducing RIP3-dependent necrosis is an attractive strategy to circumvent apoptosis resistance of cancer cells. In addition to DNA alkylating agents, which induce necrosis in a
PARP-dependent manner [50,122], some drugs have already been reported to induce RIP3-dependent necrosis [123,124]. Whereas HCC prevention strategies need to inhibit cell death at early stages to halt the HCC-promoting cell death-inflammation-regeneration-fibrosis cascade, treatment of established HCC requires promotion of cell death. As such, a better understanding of the pathophysiology and mechanisms underlying progression of liver diseases, particularly HCC, is of utmost importance.

**Table 1:** In *vivo* pathologies associated with necroptosis.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Pathology</th>
<th>Model</th>
<th>Involvement of necroptosis</th>
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<td>Chronic neurodegenerative disorders</td>
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<td>Coculture of motor neurons with astrocytes of ALS patients</td>
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<td>Aluminium-induced neurotoxicity (AD model)</td>
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<td>Cisplatin-induced AKI</td>
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4. **Currently available tools for targeting necroptosis**

No inhibitors of necroptosis are currently in clinical use. In fact, given the relatively new concept of necroptosis, only a scarce number of inhibitors have been developed, each displaying major drawbacks and/or side effects.

4.1. **Necrostatins (NECs)**

Necrostatins (NECs) are a family of compounds of diverse chemical structure that have been named for their ability to block necrotic cell death, namely by directly inhibiting the kinase activity of RIP1 [19,125]. To date, several structurally distinct NECs have been synthesized and tested as potential inhibitors of necroptosis in different experimental animal models [125,126].

Necrostatin-1 (Nec-1; 5-(1H-Indol-3-ylmethyl)-(2-thio-3-methyl) hydantoin) was identified in 2005 by Alexei Degterev and Junying Yuan as a molecule blocking necrotic cell death in human and murine cells [19]. In a subsequent study, Nec-1 was identified as an allosteric inhibitor of RIP1 kinase activity. Nec-1 is now widely used to target RIP1 kinase activity in various experimental disease models. However, several studies have raised critical concerns
regarding its specificity, appropriate control and effective concentration, particularly in murine experimental disease models. Nec-1 is a non-specific RIP1 kinase inhibitor, and also blocks indoleamine-pyrrole 2,3-dioxygenase (IDO), PAK1 and PKAα [127]. Nec-1s, a Nec-1 derivative, has specificity for RIP1 kinase over a broad range of kinases [76]. However, in particular cellular contexts, RIP1 kinase also mediates inflammatory responses and apoptosis. Moreover, Nec-1 and Nec-1s have inadequate pharmacokinetic properties, namely very short half-lives. Finally, necroptosis can occur in a RIP1-independent manner. For instance, although RIP3-deficiency protects from liver injury in an animal model of alcoholic liver disease, Nec-1 does not display any protective effects [128].

Nec-5 (2-[[3,4,5,6,7,8-hexahydro-3-(4-methoxyphenyl)-4-oxo[1]benzothieno[2,3-d]pyrimidin-2-yl]thio]-acetonitrile) also targets RIP1 during necroptosis, although through distinct mechanisms from those of Nec-1 [125]. It was reported that Nec-5 is a selective allosteric inhibitor of RIP1 that prevents the death of TNF-α-treated FADD-deficient Jurkat cells, with an EC$_{50}$ value of 240 nM [125].

Nec-7 (5-((3-(4-fluorophenyl)-1H-pyrazol-4-yl) methylene)-2-imino-3-(thiazol-2-yl) thiazolidin-4-one) is a necroptosis inhibitor that is structurally and biologically distinct from other NECs, as it does not inhibit RIP1. Nec-7 may target an additional regulatory molecule in this pathway as it inhibits TNF-α-induced necroptosis in a FADD-deficient variant of human Jurkat T cells, with an EC$_{50}$ value of 10.6μM (77).

The specificity and activity of three NEC analogs (Nec-1, Nec-5 and Nec-7) have been examined thoroughly both in vitro and in vivo, providing important information about NECs in disease models [76]. Overall, data from the literature suggests that NECs have emerged as the first-in class inhibitors of RIP1, the key upstream kinase involved in the activation of necroptosis.
4.2. Vorinostat

Histone deacetylase (HDAC) inhibitors are well-known anticancer agents possessing anti-inflammatory and neuroprotective effects. Vorinostat (suberoylanilide hydroxamic acid) was the first HDAC inhibitor to be approved by the U.S. Food & Drug Administration (FDA) for the treatment of relapsed/refractory cutaneous T cell lymphoma. Wang et al. first reported the anti-necroptotic mechanism underlying the effects of vorinostat [129]. Murine fibrosarcoma L929 cells pretreated with vorinostat (1μM) were treated with TNF-α to induce necroptosis. Vorinostat pretreatment effectively protected L929 cells against TNF-α-induced necroptosis, and this protection is conferred through mechanisms involving RIP1-dependent NF-κB and p38 MAPK activation, JNK and Akt kinase inactivation, autophagy initiation, and enhanced cFLIPL expression (a negative regulator of necroptosis). Overall, the authors show that vorinostat exerts its anti-inflammatory and cell protective effects by preventing necroptosis, an important process in inflammation and elimination of cells.

4.3. Ponatinib and Pazopanib

Ponatinib is an oral multi-targeted tyrosine kinase inhibitor developed for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. This inhibitor is used as second-line treatment for patients who have acquired resistance to standard therapy. In parallel, pazopanib is an oral receptor tyrosine kinase inhibitor approved for treatment of patients with advanced renal cell carcinoma and soft tissue sarcoma [130,131]. It targets vascular endothelial growth factor receptor (VEGFR)-1, -2, -3, platelet-derived growth factor receptor β (PDGFRβ), and c-Kit.

Fauster A et al. screened a panel of 268 FDA-approved drugs with diverse mechanisms for their ability to inhibit TNF-α-induced necroptosis in FADD-deficient human Jurkat T cells, and found that these two kinase inhibitors were potential blockers of necroptosis at submicromolar EC_{50} concentrations [132]. Both drugs inhibited necroptotic signaling triggered by various cell death receptors, whereas they did not interfere with apoptosis.
Ponatinib directly binds to and inhibits both RIP1 and RIP3. These compounds have been developed and approved as anti-cancer treatment molecules, and their safety profiles have been evaluated in this context, with both drugs reported to cause severe side effects. The definition of the cellular target spectrum might be useful in gaining a better understanding of the molecular mechanisms underlying the reported adverse effects. Thus, it still remains necessary to elucidate their exact mechanisms of action and to perform a series of careful studies in animal models covering a large variety of necroptosis-associated pathologies.

4.4. 1-Benzyl-1H-pyrazole Derivatives

Zou et al. recently screened several compounds using their in-house database and found that 1-(2,4-dichlorobenzyl)-3-nitro-1H-pyrazole exhibited potential necroptosis inhibition activity [133]. Human colon cell line HT29 was treated with TNF-α, Smac-mimetic, and zVAD-FMK. TNF-α and Smac-mimetic causes cells to undergo apoptosis by triggering the formation of a caspase-8 activating complex containing RIP1, while Z-VAD-FMK is a pan-caspase inhibitor that can switch apoptosis to necroptosis in RIP3-expressing cells upon caspase-8 inhibition [25]. The authors reported that 1-(2,4-dichlorobenzyl)-3-nitro-1H-pyrazole exhibited an EC$_{50}$ value of 1.048μM and could selectively inhibit RIP1 without influencing RIP3 kinase activation. Furthermore, the authors synthesized a number of 1-benzyl-1H-pyrazole derivatives and studied their structure-activity relationship, leading to the discovery of a potent compound 4b, which contains a new scaffold of 1-benzyl-1H-pyrazole with a Kd value of 0.078μM against RIP1 and an EC$_{50}$ value of 0.160μM in a cell necroptosis inhibitory assay. The necroptosis inhibitory activity of compound 4b was compared with that of Nec-1 (EC$_{50}$: 0.860μM), and 4b was found to be more potent [133]. The authors concluded that compound 4b exhibited considerable effectiveness in inhibiting RIP1/RIP3/MLKL signaling in intact cells and showed a good protective effect in the pancreas in the L-arginine-induced pancreatitis mouse model.
4.5. Aminoisoquinolines, pyrrolo[2,3-b]pyridines, and furo[2,3-d]pyrimidines

Harris et al. screened GSK inhibitor libraries and identified a series of type II RIP-K1 inhibitors with strong necroptosis inhibitory activity [135]. The authors revealed that three distinct series, namely 1-aminoisoquinolines, pyrrolo[2,3-b]pyridines, and furo[2,3-d]pyrimidines showed a dose-proportional response in inhibiting necroptosis and hypothermia in a mouse model of TNF-α-induced lethal shock.

4.6. Necrosulfonamide (NSA)

Due to the potential drawbacks of Nec-1, including disruption of necrosome formation, the need for a potent necrosome-downstream necroptosis inhibitor has been highlighted. Liao D et al. identified necrosulfonamide (NSA) as the first downstream small molecule inhibitor of necroptosis, by directly targeting MLKL [136]. However, this compound blocks human MLKL through the formation of a covalent bond with Cys86, but lacks activity against mouse protein due to the absence of the orthologous Cys. In this regard, it cannot be used in murine models of disease, invalidating pharmacological, pharmacokinetic and toxicity pre-clinical testing. Other MLKL inhibitors were studied in mouse models but exhibited excessive toxicity to cells and had multiple targets on other necrosome components [137]. However, research should be focused on developing new MLKL inhibitors to expand our knowledge on the in vivo significance of MLKL-dependent necroptosis in animal models. This research will serve as an invaluable tool in advancing the understanding on the significance of blocking necroptosis.

4.7. IM-54

IM-54 (1-Methyl-3-(1-methyl-1H-indol-3-yl)-4-(pentylamino)-1H-pyrrole-2,5-dione, 2-(1H-Indol-3-yl)-3-pentylamino-maleimide), a novel indolylmaleimide derivative, was shown to inhibit necrotic cell death induced by hydrogen peroxide (H2O2), but not apoptotic cell death [138]. IM-54 selectively blocks oxidative stress-induced necrotic cell death (~3μM). The
authors suggest that IM-54 is expected to constitute a powerful bioprobe for clarifying the unique signaling pathway of necrotic cell death.

4.8. NecroX analogs

NecroX are a series of indole-containing inhibitors, which were originally identified in a cell-based screen as suppressors of drug-induced necrosis in hepatocytes [139]. These molecules display general anti-oxidant and peroxynitrite (ONOO⁻) scavenging activities in vitro, and were further investigated as inhibitors of mitochondrial oxidative and nitrosative stress, which are hallmarks of pathologic necrosis [140]. One of these molecules, NecroX-1, was found to effectively inhibit toxicity caused by the pro-oxidant tBHT and reduce acute hepatotoxicity of CCL4 and streptozotocin (STZ)-induced pancreatic islet destruction [139]. Furthermore, NecroX-2 and NecroX-5 are cell permeable necrosis inhibitors with antioxidant activity. They mostly target mitochondria and selectively block oxidative stress-induced necrotic death. These analogs were also reported to protect cells against cold shock, hypoxia, and oxidative stress in vitro, as well as CCL4-induced acute liver and chronic liver fibrosis in rodent models [139,141]. Another member of this inhibitor family, NecroX-7, was shown to attenuate ischemia–reperfusion liver injury in dogs, doxorubicin induced cardiomyopathy in rats, acetaminophen-induced hepatotoxicity, non-alcoholic steatohepatitis, and allogeneic transplantation-induced graft versus host disease (GVHD) in mice [142-146]. NecroX-7 hampers release of the necrotic danger associated molecular pattern (DAMP) protein HMGB1 in vivo, and was proposed to act through inhibition of NADPH oxidase activity [143-145]. In contrast, NecroX-5 reduces myocardial hypoxia-reoxygenation injury in rats through the blockade of the mitochondrial Ca²⁺ uniporter [147]. Overall, these results highlight NecroX molecules as a useful class of mitochondria-targeting agents with putative anti-necroptotic activities.
4.9. GSK-843 and GSK-872

GSK-843 and GSK-872 were recently discovered as potent and selective RIP3 kinase inhibitors [148]. However, they are also highly toxic and strongly induce apoptosis.

4.10. Necroptosis inhibitors from natural products and isolated compounds

Ganoderma lucidium Mycelia (G. lucidum): Xuan et al. recently reported that a water-soluble extract from G. lucidum prevents necroptosis in hypoxia/ischemia-induced injury in the type 2 diabetic mouse brain [149]. G. lucidum is a very popular medicinal fungus used in traditional Chinese medicine; it has an extensive variety of pharmacological activities, including antioxidant, anticancer, anti-inflammatory, and immunomodulatory activities [150].

Terminalia Chebula (T. chebula): A recent report from Lee et al. reported that T. chebula water extract (WETC) provides protection against necroptotic cell death upon death receptor engagement [151]. WETC was found to antagonize mitochondrial-derived ROS production and, as such, inhibit TNF-induced necroptotic cell death.

Kongensin A (KA): Li et al. have recently reported that KA, a natural product isolated from Croton kongensis, shows unique signaling mechanisms and is capable of inhibiting necroptosis [152]. The authors suggest that KA blocks necroptosis before or at the RIP3 activation step.

Celastrol: Celastrol is a triterpene from the root bark of the Chinese medicinal plant Tripterygium wilfordii that was recently reported to inhibit necroptosis and alleviate ulcerative colitis in mice [153]. Celastrol was shown to exerted beneficial effects in the treatment of colitis by suppressing the RIP3/MLKL necroptosis pathway.
Naringenin (NGEN): Chtourou et al. reported that Naringenin (NGEN) protects against cardiac hypercholesterolemia-induced oxidative stress and subsequent necroptosis in rats [154]. NGEN was shown to exert anti-inflammatory effects in the liver, and long-term (12 weeks) treatment with NGEN inhibits high cholesterol diet (HCD)-induced fibrosis in the rat liver [155].

Curcumin: Curcumin is the principal curcuminoid of Curcuma longa (turmeric), a member of the ginger family (Zingiberaceae). While earlier studies have suggested that curcumin attenuates neuronal apoptosis, Dai et al. recently showed that the neuroprotective effects of curcumin also result from attenuation of the necroptosis pathway, upon co-incubation with z.VAD.fmk to block apoptosis in cultured primary cortical neurons injured with ferrous chloride. Curcumin (~10μM) decreased expression of RIP1 in a dose- and time-dependent manner [156].

A summary of various necroptosis inhibitors is depicted in Table 2.

Table 2: Summary of necroptosis inhibitors.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Compound</th>
<th>Mode of action</th>
<th>Ref.s</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Necrostatins" /></td>
<td><strong>Necrostatins</strong>&lt;br&gt;Nec-1, Nec-2, Nec-3, Nec-1s&lt;br&gt;7-Cl-Nec-1, 7-Cl-O-Nec-1&lt;br&gt;R-7-Cl-O-Nec-1&lt;br&gt;Nec-5, Nec-7</td>
<td>RIP1 inhibitor</td>
<td>[77,125-128]</td>
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<tr>
<td><img src="image2.png" alt="Vorinostat" /></td>
<td><strong>Vorinostat</strong></td>
<td>RIP1 inhibitor</td>
<td>[129]</td>
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<tr>
<td><img src="image3.png" alt="Ponatinib" /></td>
<td><strong>Ponatinib</strong></td>
<td>RIP1/3 inhibitor</td>
<td>[132]</td>
</tr>
<tr>
<td><img src="image4.png" alt="Pazopanib" /></td>
<td><strong>Pazopanib</strong></td>
<td>RIP1 inhibitor</td>
<td></td>
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<tr>
<td><img src="image5.png" alt="1-Benzyl-1H-pyrazole Derivatives" /></td>
<td><strong>1-Benzyl-1H-pyrazole Derivatives</strong></td>
<td>RIP1 inhibitor</td>
<td>[25,133]</td>
</tr>
<tr>
<td>Aminoisquinolines, Furo[2,3-d] pyrimidines Pyrrolo[2,3-b] pyridines</td>
<td>RIP1 inhibitor [135]</td>
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<tr>
<td><strong>Necrosulfonamide</strong></td>
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<td><strong>IM-54</strong></td>
<td>Necroptosis-related ROS inhibitor [138]</td>
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<td><strong>NecroX analogs</strong></td>
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<td>GSK‘843</td>
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<td>GSK‘872</td>
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<td><strong>Natural products and isolated compounds</strong></td>
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<tr>
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<td>Curcumin</td>
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5. Identification and validation of protein targets of bioactive small molecules

The first step in the discovery of new bioactive small molecules is the selection of biologically active compounds among abundant sources of small molecules. In chemical biology, there are two basic approaches to identify the mode of action of small molecules in determined biological systems [157]. In target-based screening, which can be summarized as “from target to phenotype”, the process starts with a functionally validated target protein;
specific small molecules that can modulate the activity of the target are then screened, after which the cellular phenotypic changes caused by perturbation of the target protein are analyzed. The other approach is phenotype-based screening, defined as “from phenotype to causative target”; in this approach, a large proportion of small molecules of interest are identified using phenotypic screens in cells or whole organisms [158,159]. A specific cellular phenotype caused by a certain small molecule reflects the presence of a functional target of the small molecule in the cells; this target protein may be a key mediator of the phenotypic change caused by the small molecule in a certain disease model.

Identification of the molecular targets of bioactive small molecules is a crucial step in both chemical biology research and drug development [160-162]. The interaction between the small molecule and its target protein is key to understand the cellular mechanism(s) by which the small molecule acts. However, within the organism, small molecules can affect more than one protein or signaling pathway, and may cause undesired biological activities or toxicity by interacting with off-target proteins [163]. In contrast, a small molecule may owe its biological activity to simultaneous targeting of several proteins [164]. Therefore, the deconvolution of both therapeutic- and off-targets is essential for the development of drugs with superior pharmacological properties and fewer side effects. However, identifying direct targets is a time-consuming and challenging step, because a standardized methodology that can be successfully applied to most cases is yet to be established. Nevertheless, several methods for target identification based on genomics, proteomics, and bioinformatics approaches have been developed, and several functional target proteins of bioactive small molecules have been identified (Table 3) [165].

<table>
<thead>
<tr>
<th>Approach</th>
<th>Method</th>
<th>Small molecule</th>
<th>Biological activity</th>
<th>Identified target</th>
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Abbreviations of methods: MSP = multicopy suppression profiling; HIP/HOP = haploinsufficiency profiling/homozygous profiling; DARTS = Drug affinity responsive target stability

6. Bile acids and cell death

Bile acids (BAs), the major constituents of bile, are produced in the liver and secreted into the intestine, where they play crucial biological roles such as solubilization of lipids in the intestinal lumen, among many others. Interestingly, the intermediates and end-products of bile acid pathways modulate the expression of genes involved in the synthesis of cholesterol, fatty acids and BAs themselves, mainly by interacting with DNA-binding nuclear receptors [191]. The primary BAs derived from bile acid biosynthesis are usually conjugated with...
glycine or taurine, being then secreted via the bile ducts into the small intestine. There, BAs act mainly as detergents to emulsify dietary lipids, due to their amphipathic structure that allows them to be water-soluble and capable of inserting themselves into biological membranes. After uptake of emulsified nutrients, BAs are re-absorbed to the liver via the portal vein, and are re-directed to the gallbladder for storage until the next feeding cycle [192]. The primary bile acid pool that enters the small intestine can suffer biotransformation catalyzed by intestinal bacteria, yielding secondary and tertiary BAs, such as ursodeoxycholic acid (UDCA) [193].

Certain BAs are cytotoxic molecules, implicated in cancer development in the intestinal tract [194] and/or cell death by necrosis and apoptosis. In fact, apoptosis has been described as a key event during hepatobiliary diseases [195]. Curiously, not all BAs are cytotoxic, which may be related to minor changes in their chemical structure [196]. In this regard, accumulation of hydrophobic BAs within the hepatocyte induces cell death of liver cells during cholestasis, while hydrophilic BAs, such as, UDCA are cytoprotective in different cellular contexts [197].

UDCA is a hydrophilic bile acid used for the treatment of patients with hepatobiliary disorders, being widely considered as the first choice therapy for chronic cholestatic diseases [198]. The cytoprotective properties of UDCA derive from its ability to abrogate apoptosis, although the molecular mechanisms engaged in this response are still not entirely unraveled. It is known that it involves stabilization of mitochondrial membranes, decreased Bax translocation into the mitochondria and reduced opening of the permeability transition pore, which further restricts cytochrome c release and subsequent caspase activation and substrate cleavage [199-202]. Further, UDCA has been demonstrated to strongly modulate the expression of several genes involved in apoptosis, cell cycle regulation and proliferation in hepatocytes [203].

Tauroursodeoxycholic acid (TUDCA) is an endogenous hydrophilic BA that derives from the conjugation of UDCA with taurine [197]. TUDCA showcases strong anti-apoptotic properties in multiple cellular contexts, including several experimental models of
neurodegeneration. TUDCA is also a potent inhibitor of ER stress-mediated pathways, reducing calcium efflux and activation of caspase-12 [204,205].

Given the anti-apoptotic mechanisms discovered for BAs and its promising results on the treatment of hepatic and neurodegenerative diseases, research on their possible effects in other cell death pathways, namely necroptosis, is ongoing. However, no studies have yet identified a direct link between BAs and necroptosis. Zhangxue H et al. demonstrated that BAs induce apoptosis at low concentrations but induce necroptosis at high concentrations. BAs maybe have a direct effect on death-inducing signaling complex (DISC), a pathway that also activates necroptosis, and it appears that they may upregulate RIP3 expression via FXR [206]. Still, more investigation is needed in this field.
Objectives

Necroptosis plays a key regulatory role in various physiological processes and is found unbalanced in several human diseases. However, no inhibitors of necroptosis are currently in clinical use.

The overall goal of this study was to identify novel modulators of necroptosis and to elucidate their mechanism(s) of action. In order to achieve this goal, several intermediate milestones were envisaged:

(A) Evaluation of BAs as potential modulators of necroptosis:
1. Biological screening of 6 BAs and 4 newly synthesized derivatives (provided by Prof. Jorge Salvador, University of Coimbra), with selection of hits;
2. Hit confirmation/validation with EC_{50} determination;

(B) Evaluation of a group of novel small molecules as potential modulators of necroptosis:
1. Biological screening of 21 newly synthesized small molecules (provided by Prof. Carlos Afonso, Faculty of Pharmacy), with selection of hits;
2. Hit confirmation/validation with IC_{50} and EC_{50} determination;
3. Target identification and confirmation by Western Blot analysis;
4. In silico docking studies of selected hits.

The identification of specific modulators will help in the design of novel and more effective therapeutic strategies for human pathological conditions caused or aggravated by necroptotic cell death.
Materials and Methods
Cell culture, reagents and antibodies

L929 cells were cultured in DMEM (GIBCO®, Life Technologies, Inc.; Grand Island, USA) supplemented with 10% FBS and 1% Glutamax (GIBCO®). The reagents used were as follows: mouse TNF-α (PeproTech EC Ltd., London, UK), Necrostatin-1 (Sigma-Aldrich Co., St Louis, MO, USA) and DMSO (Sigma-Aldrich Co.). The following antibodies were used: β-actin (Sigma-Aldrich Co.), RIP1 (Cell Signaling, Inc., Danvers, MA, USA), phospho-MLKL and total MLKL total (Sigma-Aldrich Co.). The secondary antibodies used were goat anti-mouse HRP and goat anti-rabbit HRP (BioRad Laboratories, Hercules, CA, USA).

A library of twenty-one small molecules potentially inhibitors of necroptosis (OXA compounds) were synthetized and gently provided by Prof. Carlos Afonso, Faculty of Pharmacy of University of Lisbon. BAs were purchased from Sigma-Aldrich Co. A small library of BA derivatives (SB compounds) was synthetized and gently provided by Prof. Jorge Salvador, University of Coimbra. The four most promising SB compounds, in terms of necroptosis inhibition, were pre-selected based on a cell viability screen performed by Hugo Brito, PhD student in the group. These were SB3, a CA derivative; SB12, a CDA derivative; SB16, a UDCA derivative; and SB22, a TUDCA derivative.

Chemical screening

L929 cells were plated in solid clear bottom 384-well plate at 1000 cells per well (15μl) in DMEM medium supplemented with 1% Glutamax. After 24h, TNF-α and test compounds were added (30 μM) at each plate, in duplicated. Appropriate controls were used, namely DMSO control wells, as well as Nec-1 (30 μM) and TNF-α (30 μM) isolated wells. After 6h, general cell death was determined using 10μL of culture media from each well, mixed with 10μL of bioluminescent cytolysis assay, in order to measure the release of adenylate kinase enzyme from damaged cells (ToxiLight™ BioAssay Kit, Lonza). The same procedure was followed when screening SB compounds (30, 60 and 100μM). CA, DCA, CDCA, OCA, UDCA and TUDCA were used as controls.
Viability assay

Cell viability of L929 cells was analyzed using MTS assay. The assay is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The formazan dye produced by viable cells can be quantified by measuring the absorbance at 490-500 nm.

Briefly, L929 cells were seeded in 96-well plates at a density of 5000 cells per well and, after 24h, incubated with selected compounds at ten different concentrations (1, 5, 10, 20, 30, 60, 90, 150, 200 and 300 µM) in duplicate. After 24h or 48h, the medium was aspirated, the MTS solution added and the absorbance recorded at 490nm for each well on the GloMax-Multi+Detection System (Promega Corp.). Compound toxicity was assessed by determining the half maximal inhibitory concentration (IC$_{50}$) from the dose-response curves.

Half maximum effective concentrations (EC$_{50}$), in inhibiting necroptosis, were also determined for select hits, based on dose-response curves in L929 cells. Briefly, 24h after plating, L929 cells were incubated with selected compounds at nine different concentrations (0.5, 1, 5, 10, 17, 25, 30, 40 and 50 µM) plus TNF-α (30µM) in duplicate. After 6h, release of adenylate kinase enzyme from damaged cells (ToxiLight™ BioAssay Kit, Lonza) was measured, as previous described. For BAs and its derivatives, a different range of concentrations in culture was used, namely 5, 10, 30, 60, 100, 150, 200, 300 and 400 µM.

Microscopy

L929 cells morphology was evaluated by phase-contrast microscopy using a Primo Vert microscope (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Images were acquired under 100x magnification using an AxioCam 105 Color camera with the ZEN lite 2012 (both from Carl Zeiss MicroImaging GmbH).
**Total Protein Isolation**

For total protein isolation, L929 cells were plated in 6-well plates at 250.000 cells per well. After 24h, cells were incubated with compounds in the presence or absence of TNF-α. After 7h, the medium was discarded and cells were collected to a tube after treatment with Tryple (~500 µl) during 5min at 37ºC. The wells were washed with medium (~500 µl) and collected to the previous tube. The tubes were then centrifuged for 4min at 600g (4ºC). Finally, the supernatant was completely removed and discarded. The cell pellet was homogenized in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM dithiothreitol) and 1x Halt Protease and Phosphatase Inhibitor Cocktail (Pierce, Thermo Fisher Scientific). After keeping on ice for 30 min, the samples were sonicated for 30sec in ultrasounds (sonication adjusted to 80% amplitude and 90% pulse – model UP100H, Hielscher Ultrasonics GmbH – 100 watts, ultrasonic frequency 30 Hz). The lysate was centrifuged at 3,200 g for 10 min at 4ºC and the supernatant recovered, and stored at -80ºC.

**Western Blot Analysis**

Total protein extracts from L929 cells (see above) were used. Protein concentrations were calculated using the Bio-Rad protein assay kit, according to the manufacturer’s recommendations. Equal amounts of protein (50 µg) were electrophoretically resolved on denaturing 8% polyacrilamide gels. The resolved proteins were transferred onto nitrocellulose membranes and blocking was performed with a 5% milk solution. Membranes were then incubated overnight with the primary antibodies. In the following day, membranes were incubated with goat secondary antibodies conjugated with horseradish peroxidase anti-mouse or anti-rabbit for 3h at room temperature. After rinsing with TBS three times (10 min each), the immunoreactive proteins were visualized with Immobilon™ Western (Millipore) or SuperSignal West Femto substrate (Thermo Fisher Scientific Inc). β-actin and Ponceau S
staining were used as loading controls. Densitometric analyses were performed with the Image Lab software Version 5.1 Beta (Bio-Rad).

**Statistical Analysis**

All data are expressed as the mean ± SEM from at least three independent experiments. Statistical significance was evaluated using the Student’s t-test. \( p < 0.05 \) was considered statistically significant.
Results and Discussion
(A) Evaluation of BAs as potential modulators of necroptosis

1. Biological screening of 6 BAs and 4 newly synthesized derivatives

To evaluate whether BAs are capable of inhibiting necroptosis, L929 cells were incubated with naturally occurring BAs (CA, DCA, CDCA, UDCA and TUDCA) or its derivatives (OCA, SB3, SB12, SB16 and SB22), in the presence or absence of TNF-α, as described in Materials and Methods and illustrated in Figure 2.

Results showed that TNF-α-induced AK release, an indirect measure of TNF-α-induced necroptosis, was significantly prevented upon co-incubation with Nec-1 (p < 0.01; Figure 3). In contrast, the bile acids CA, DCA and CDCA failed to inhibit TNF-α-induced necroptosis (Figure 3) and were cytotoxic when incubated alone (30-100 µM; data not shown). As such, these BAs were not used in subsequent experiments.

We next evaluated whether more hydrophilic BAs, UDCA and TUDCA, as well as novel BA derivatives, were able to inhibit TNF-α-induced necroptosis, in a dose-dependent manner. L929 cells were co-incubated with 30 µM TNF-α and either 30, 60 or 100 µM of BA species for 6 h.
Figure 3 - CA, DCA and CDCA fail to modulate TNF-α-induced L929 cell necroptosis. L929 cells were incubated with 30 µM TNF-α in the presence or absence of either 30 µM Nec-1, CA, DCA, or CDCA. Necroptosis was determined using the ToxiLight™ BioAssay Kit. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates. N/A, no addition. §p < 0.05 and *p < 0.01 vs. N/A; ‡p < 0.01 vs. TNF-α.

Of note, some compounds were already cytotoxic when incubated alone, in a dose-dependent manner, particularly SB12 (p < 0.01 for 30, 60 and 100 µM) and SB3 (p < 0.01 for 60 and 100 µM) (Figure 4). In addition, they completely failed to prevent, or even slightly increased TNF-α-induced cytotoxicity. Because SB12 and SB3 are CDA and CA derivatives, respectively, these results suggest that the structural modifications made to the original bile acids had no effects on its ability to inhibit cell death.

In turn, more hydrophilic BAs UDCA and TUDCA, as well as its derivatives SB16 and SB22, respectively, exhibited low cytotoxicity when incubated alone, even at the highest 100 µM concentrations. The same effect was observed for OCA. In addition, the original UDCA and TUDCA scaffolds were effective against necroptosis when at 100 µM (p < 0.01 and p < 0.05, respectively). OCA was also effective at 100 µM; SB16 at 30 µM; and SB22 at 100 µM (all p < 0.01) (Figure 4, Suppl. Figure 1).
2. Hit confirmation/validation

Unfortunately, we were unable to continue our studies with the SB16 and SB22 derivatives, due to their very limited availability. To further characterize the activities of OCA, UDCA and TUDCA, we next determined the half maximal effective concentration (EC\textsubscript{50}) for inhibiting necroptosis using concentrations ranging from 5 to 400 μM. Results showed that all three BAs were able to inhibit TNF-α-induced necroptosis and OCA had an EC\textsubscript{50} >50 μM (Figure 5), a very high value for an optimal small molecule inhibitor. In addition, and oddly, we were unable to calculate the EC\textsubscript{50} values for UDCA and TUDCA. As such, a larger range of concentrations should be used in the future. In particular, because OCA, UDCA and TUDCA were effective against necroptosis at the 100 μM concentration, but not at 30 or 60 μM, and exhibited low cytotoxicity when incubated alone at all tested concentrations. Higher concentrations > 400 μM could be tested. Similarly, it could be worthwhile to synthesize more SB16 and SB22 compounds for calculation of EC\textsubscript{50} values since, as they inhibited TNF-α induced necroptosis at 100 μM and were not cytotoxic at this maximal concentration. Still, as EC\textsubscript{50} values increase, the likelihood of compounds depicting a good small molecule inhibitor
decreases. For this reason, we have not proceeded with a more detailed study on the mechanisms of action of these bile acids in inhibiting necroptosis.

**Figure 5– Dose response curve showing EC50 of OCA, UDCA and TUDCA on inhibition of L929 cell viability.** L929 cells were incubated with 30 μM TNF-α in the presence of concentrations ranging from 5 to 400 μM BAs. Necroptosis was determined using the ToxiLight™ BioAssay Kit. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates.
(B) Evaluation of a group of novel small molecules as potential modulators of necroptosis

1. Biological screening of 21 newly synthesized small molecules

A chemical library of 21 newly synthesized small molecules, some with structural similarities to Nec-1, was tested for their ability to block TNF-α-induced cell death in L929 cells, aiming at the identification of novel inhibitors of necroptosis (Suppl. Table 1). L929 cells were incubated with compounds, in the presence or absence of TNF-α, as described in Materials and Methods and as illustrated in Figure 2. Nec-1 was used as a positive control [19].

Results showed that TNF-α-induced AK release, an indirect measure of TNF-α-induced necroptosis, was almost completely abrogated upon co-incubation with Nec-1 (p < 0.05; Figure 6). Among the compounds investigated, OXA003, 012, 014 and 017 robustly rescued cell death (p < 0.01, Figure 6). Noteworthy, OXA012, 014 and 017 appeared to afford similar cytoprotection compared with Nec-1.

Figure 6 - OXA003, OXA012, OXA014 and OXA017 inhibit TNF-α-induced L929 cell necroptosis. L929 cells were incubated with 30 µM TNF-α in the presence or absence of either 30 µM Nec-1 or...
OXA molecules. Necroptosis was determined using the ToxiLight™ BioAssay Kit. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates. N/A, no addition. *p < 0.01 vs. N/A; †p < 0.05 and ‡p < 0.01 vs. TNF-α.

We next validated our results for different lots of OXA003, OXA012, OXA014 and OXA017 compounds. A newly synthesized OXA compound - OXA019 - was evaluated in parallel. Two different lots of OXA012 and one different lot of OXA003, OXA014 and OXA017 were able to inhibit TNF-α-induced necroptosis in a similar range as before (p < 0.05 for OXA012 and OXA017; and p < 0.01 for OXA033 and OXA014; Figure 7), proving the robustness and consistency of the synthesis process. The newly synthesized OXA019 was not cytotoxic at 30 µM (p < 0.01) and, noteworthy, significantly inhibited TNF-α-induced necroptosis more efficiently than the remaining compounds or even Nec-1 (p < 0.01).

![Figure 7](image_url)

Figure 7 - Different lots of OXA003, OXA012, OXA014 and OXA017, as well as a newly synthesized OXA - OXA019 - completely prevent TNF-α-induced L929 cell necroptosis. L929 cells were incubated with 30 µM TNF-α in the presence or absence of either 30 µM Nec-1 or OXA molecules. Necroptosis was determined using the ToxiLight™ BioAssay Kit. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates. N/A, no addition. *p < 0.01 vs. N/A; †p < 0.05 and ‡p < 0.01 vs. TNF-α.
2. Hit confirmation/validation

To further characterize the activities of our identified five hits, we performed dose-response curves to quantitatively assess its inhibitory potency. The EC$_{50}$ for inhibiting necroptosis was determined using concentrations ranging from 0.5 to 50 µM (Figure 8). Results showed that, with the exception of OXA003, selected hits were able to inhibit TNF-α-induced necroptosis with an EC$_{50}$ below 25 µM (Figure 8).

![Dose response curve showing EC50 of OXA003, OXA012, OXA014, OXA017 and OXA019 on inhibition of TNF-α-induced L929 cell death. L929 cells were incubated with 30 µM TNF-α in the presence of concentrations ranging from 0.5 to 50 µM OXAs. Necroptosis was determined after 6 h, using the ToxiLight™ BioAssay Kit. The dotted line represents total reversion of cell death. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates.](image)

In the previous assay, our results suggested that OXA019 (30µM) was more effective at fully inhibiting TNF-α-induced necroptosis, when compared with Nec-1 (30 µM). Despite still being the most potent inhibitor of necroptosis among the 5 OXAs, it is now clear that Nec-1 remains the most effective inhibitor of necroptosis, in terms of half maximum effective concentration (8.73x10$^{-7}$ µM vs 7.42 µM for OXA019). Results further showed that the remaining OXAs display similar dose-response curves and EC$_{50}$ values, with the exception of OXA003, which failed to completely inhibit cell death at any of the tested concentrations (EC$_{50}$ > 50 µM).
The citotoxicity of selected hits in L929 cells was assessed by determining the half maximal inhibitory concentration (IC$_{50}$) using concentrations ranging from 1 to 300 μM, and a period of incubation of 48 h. Cell viability was assessed using the MTS metabolism assay. At 300 μM, the highest concentration used, only OXA003 induced cell death by more than 50% (IC$_{50}$ = 28.6 μM; Figure 9). OXA017 was also slightly cytotoxic at this concentration (IC$_{50}$ = 114.5 μM). OXA012, OXA014 and OXA019 displayed no cytotoxicity throughout the all range of tested concentrations (Figure 9), with IC$_{50}$ values greater than 300 μM, highlighting the window of opportunity for inhibiting necroptosis with this particular set of compounds.

![Graph](image)

**Figure 9** – OXA012, OXA014 and OXA019 are not toxic to L929 cells in a wide range of concentrations. L929 cells were incubated with concentrations ranging from 1 to 300 μM OXAs. Cell viability was determined after 48 h using the MTS assay. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates.

We also compared the EC50 of hits with that of Nec-1 after 24 h in culture. Results show that after 24 h, Nec-1 still protects L929 cells from TNF-α-induced cell death, near to 100%, throughout the whole range of tested concentrations (Figure 10). In turn, despite the previous evidence of full protection from necroptosis by OXA012 and OXA014, at 6 h of incubation (Figures 6 and 7), protection dropped to approximately 20% after 24 h (Figure 10; OXA019 was not available at the time of this experience). In line with OXA003 and OXA017 IC$_{50}$ values (Figure 9), both failed to significantly prevent necroptosis after 24h in culture (Figure 10).
Figure 10 – All OXA compounds display reduced protection from TNF-α-induced necroptosis with time. L929 cells were incubated with 30 μM TNF-α in the presence of concentrations ranging from 1 to 300 μM OXAs. Cell viability was determined after 24 h using the MTS assay. The upper dotted line represents linearized N/A results (100% viability), while the lower dotted line represents linearized TNF-α results (100% cell death). Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates.

Because OXA003 was the least effective among the 5 selected OXAs in inhibiting TNF-α-induced necroptosis, and due to its inherent cytotoxicity, it was excluded from subsequent experiments.

We next aimed to validate the cytoprotection of OXA012, 014, 017 and 019 from TNF-α-induced necroptosis using phase-contrast microscopy analysis (Figure 11). Compared to cells incubated with TNF-α, where pronounced cell death is evident, OXA012, OXA 014, OXA017 and OXA019 appear to protect L929 cells from TNF-α-induced death, particularly OXA 014, OXA017 and OXA019; cells incubated with TNF-α in the presence of these compounds are present at a higher density/power field, when compared with TNF-α alone, and its morphology more closely resembles that of control (N/A) cells. Of note, this microscopic analysis also allowed us to visualize that compounds form precipitate agglomerates at the 30 μM concentration, indicating that the solubility proprieties of these compounds should be improved. If so, it is possible that their anti-necroptotic properties may also further improve.
3. Target identification and confirmation

**OXA012 and OXA019 inhibit RIP1 protein expression and MLKL activity**

Given that RIP1 and MLKL are key players of necroptosis, we set out to assess which, if any, embodied a target of the selected hits. L929 cells were incubated with 30 μM TNF-α (or no addition) in the presence or absence of 30 μM OXA012, OXA014, OXA017 and OXA019 for 24 h. 30 μM Nec-1 was used as a positive control. Results showed that TNF-α induced RIP1 and MLKL phosphorylation by ~ 50 and 100%, respectively ($p < 0.01$ for both; Figure 12). Nec-1 completely halted both RIP1 and phosphorylated MLKL (pMLKL) expression ($p < 0.01$ for both). Despite the fact that all OXA compounds inhibited TNF-α-induced RIP1 and MLKL phosphorylation to a certain degree, only OXA012 and OXA019 were able to do so in a statistically significant manner. In particular, OXA019 inhibited RIP1 ($p < 0.01$) and pMLKL ($p < 0.05$) by ~ 70% and 50%, respectively.
Taken together, the inhibition of necroptosis by the OXA compounds may involve direct or indirect targeting of both RIP1 and MLKL.

Figure 12 - OXA012 and OXA019 inhibit TNF-α induced RIP1 and MLKL phosphorylation. L929 cells were incubated with 30 µM TNF-α (or no addition) in the presence or absence of either 30 µM OXA012, OXA014, OXA017 or OXA019 for 24 h. 30 µM Nec-1 was used as a positive control. Total protein fractions were prepared for Western blot analysis of RIP1 (A), MLKL and p-MLKL (B). Representative immunoblots are shown. Blots were normalized to Ponceau S staining. Results are presented as the mean value ± SEM for at least three independent experiments. §p < 0.05 and *p < 0.01 vs. N/A; †p < 0.05 and ‡p < 0.01 vs. TNF-α.
4. In silico docking studies of selected hits suggest that OXA012 may constitute a promising modulator of necroptosis

To explain the observed biological activities of this new series of leads and to get insight into their mechanism of action at the molecular level, OXA012 was chosen for in silico molecular docking calculations inside the RIP1 active site (using for this the X-ray structure obtained for this enzyme complexed with 1-aminoisoquinoline inhibitor at resolution of 2.57Å, PDBID: 4NEU). Docking studies were conducted by Prof. Rita Guedes from the Medicinal Chemistry group within iMed.ULisboa.

All the docking calculations were carried out with GOLD 5.2 software. Docking calculations revealed that without any constrain (e.g. a covalent bond), OXA012 is occupying a pose similar to the co-crystallised inhibitor with the phenyl rings from both compounds almost superposed, thus suggesting possible similar interaction (Figure 13A). However, OXA012 is slightly rotated compared with the crystallographic ligand, positioning this compound close to Asp156, Leu157, Met67 and Met95 (that could enable important hydrogen bonds and π interactions, however showing interaction distances increased when compared with the crystallographic inhibitor (Figure 13B).

Figure 13: In silico molecular docking calculations for OXA12. (A) Optimal poses obtained inside RIP1 active site for compound 12 compared with crystallographic ligand (PDBID: 4NEU); (B) Compound 12 and 4NEU co-crystallised inhibitor interacting with Asp156, Leu157, Met67 and Met95.
Conclusion and Future Perspectives
Regulated necrosis or necroptosis, an immunogenic programmed cell death type, morphologically similar to necrosis, has been recently implicated in disease pathogenesis. This pathway was initially identified following a cell based screen in which Degterev et al. identified a series of small molecules, termed Necrostatins, that blocked the necrotic death of human monocytic U937 cells induced by treatment with TNF-α and the caspase inhibitor zVAD.fmk [19]. These were subsequently identified as RIP1 kinase inhibitors [125] and were shown to have efficacy in animal models of IR injury in the brain, retina, and kidney, as well as models of myocardial infarction and retinal detachment [62-70]. Recent data from genetically manipulated mouse models has further highlighted the role for RIP1-dependent necroptosis as a key driver of the pathogenesis of inflammation and disease in the intestine and the skin [207-209]. Likewise, necroptosis is arising as a likely pathological feature of inflammation-driven liver diseases. For instance, RIP3 mediates ethanol or drug-induced liver injury in vivo [29,34,128], whereas apoptosis and RIP3-dependent necroptosis are simultaneously activated in an animal model of chronic hepatic inflammation [106]. In addition, concanavalin A-induced hepatic failure is associated with hepatocyte necroptosis and aberrant TNF-α signaling [105,211]. Curiously, as a potent proinflammatory cytokine, TNF-α is involved in the pathogenesis of a broad range of liver diseases, including viral hepatitis, alcoholic hepatitis, acute liver failure and NAFLD [212].

Necrostatins have moderate potency and poor pharmacokinetic properties, rendering them unsuitable for development as therapeutics. Other tool compounds blocking necrotic cell death by targeting RIP1, RIP3 and MLKL have been developed; however, and to this date, no necroptosis inhibitors are in clinical use. In order to identify novel modulators of necroptosis, we performed a biological screen using two different libraries. The first consisted of selected bile acids and newly, novel synthesized derivatives. The second comprehended twenty-one novel small molecules.

In the first library, we evaluated the ability of six bile acids and four newly synthesized derivatives to inhibit TNF-α-induced L929 cells necroptosis. It is well known that the cytoprotective properties of BAs derive in part from their ability to abrogate apoptosis,
although emerging evidence suggests that they may also interact with signaling mechanisms involved in necroptosis [206]. In fact, UDCA and TUDCA were shown to possess some anti-necroptotic activity. From the novel derivatives, only SB16 and SB22, UDCA and TUDCA derivatives, respectively, showed some modest activity. However, OCA EC\textsubscript{50} was relatively high, while the EC\textsubscript{50} values of UDCA and TUDCA, though not determined, are extrapolated to be even higher. For this reason, we have not proceeded with a more detailed study on the mechanisms of action of these bile acids in inhibiting necroptosis.

Among the twenty-one new compounds of the second library, our screen identified four hits, namely OXA012, OXA014, OXA017 and OXA019, as capable of blocking necroptosis in L929 cells at micromolar EC\textsubscript{50} concentrations. Structurally, selected compounds show some similarity when compared to Nec-1 (Suppl. Table 1); although OXA003 seemed promising at first, it turned out to be the least potent in inhibiting TNF-α-induced necroptosis, as well as the more cytotoxic. For these reasons, it was excluded from subsequent experiments. These results are consistent with chemical structures, the most different from Nec-1 from all potential selected compounds. On the other hand, OXA012, 014, 017 and 019 are structurally similar to each other, all with a benzimidazole group linked with a large ramified group. This benzimidazole group is similar to the indol group of Nec-1, with one additional nitrogen. The larger structure could be essential for interaction with targets. For instance, the structure of OXA012 allows it to interact with RIP1 active site and to establish hydrogen bonds and π interactions, which may, in turn, be responsible for its inhibition ability.

Regarding the signaling mechanisms by which these molecules modulate necroptosis, our data further pointed to a likely modulation of RIP1 and/or MLKL. Of note, RIP3 should also be evaluated as another possible target in future studies, as this kinase represents a crucial mediator of necroptosis and its protein expression levels correlate with cell sensitivity to necroptosis [73]. Additionally, the effects of RIP1 kinase activity are not limited to cell death, as RIP1 kinase activity has also been implicated as a direct driver of proinflammatory cytokine production or NF-κB-mediated cell survival [210]. Furthermore,
under certain conditions, RIP1 could also be dispensable for necroptosis or even can act as an inhibitor of this process [214]. Kearney CJ et al. demonstrated that RIP1 knockdown unexpectedly potentiates necroptosis and, in contrast, RIP3 knockdown potently suppresses necroptosis under the same conditions. As such, studies related to necroptosis inhibition must also include the evaluation of RIP3 levels to ensure that conclusions are specific of this cell death pathway.

In order to identify additional targets for the selected compounds, alternative methods could also be used, namely, drug affinity responsive target stability (DARTS). This proteomic approach is based on the principle that binding of drugs will stabilize target proteins, either globally or locally, for example, in a specific conformation or simply by masking protease recognition sites, thereby reducing the protease sensitivity of the target protein. Compared to affinity chromatography (AC), in which a small molecule-immobilized affinity matrix is used to identify target proteins from cellular or tissue extracts and utilize positive enrichment by selectively accumulating the target proteins for tag bioactive compounds of interest along with precluding non-specific binding proteins, DARTS makes use of negative enrichment by digesting away non-target proteins while leaving behind the target proteins. The DARTS technique involves separation of ligand-treated and control protein samples digested with varying amounts of protease by SDS–PAGE and analysis of each lane of the gel for bands that are more intense than the others. The key advantage of DARTS is that is does not require chemical modification of bioactive compounds of interest for binding with the protein libraries. As such, it is not limited by chemistry and can potentially be used for any small molecule. In addition, DARTS does not require extensive washing conditions and has a potential to be applicable to lower affinity interactions between the bioactive compounds of interest and their target proteins. Still, and similarly to conventional AC system, affinity of the bioactive compound to its target protein may be a limiting factor. Additionally, protein susceptibility to proteolysis might be a problematic point in DARTS [165].

The specificity of selected compounds to block necroptosis as opposed to apoptosis should also be considered in the future. Necroptosis, not apoptosis, is a key mediator of cell
loss and DAMP-mediated inflammation. In this regard, DAMPs have been described as a link between cell death and inflammation in multiple diseases, like chronic liver disease \cite{114,213}. For this reason, it is very important to find specific necroptosis inhibitors capable of attenuating inflammation-mediated tissue injury.

Apoptosis-specific assays, such as analysis of mitochondrial cytochrome c release, DNA fragmentation, and caspase activation, useful in precluding modulation of this pathway, are thus due. The annexin V/PI assay constitutes another example of a simple approach to differentiate apoptosis from necroptosis, and could prove a useful method to further characterize these novel molecules. Finally, to clarify if selected compounds are able to modulate necroptosis in particular pathologies, for instance liver diseases, it would also be important to perform hit confirmation/validation assays using liver-derived cells, including, for instance, human hepatoma cell line HepG2 and/or primary human hepatocytes.

An additional logical next step in this study would be for a chemistry team to assess structure activity relationships (SAR) of identified hits, especially OXA012 and OXA019. A SAR assessment of validated screening hits serves as the basis to design new chemical probes with improved potency. For the synthesis of new RIP1/3 and MLKL hit derivatives, methodologies involving microwaves and solid-phase catalysis to overcome possible problems with classical methods yields should be planned. In addition, the synthesis of target molecules should preferably use easily accessible key intermediates. Modifications on the scaffold should be guided by use of \textit{in silico} docking. In this way, a large number of structural motifs can be obtained and allow the development of a SAR study based on results obtained in the biological activity studies. Additionally, SAR assessment also considers the physical properties of the compounds, including predictions of membrane permeability, solubility, toxicity (e.g. based on established chemical “signatures” for reactivity) and synthetic route planning for new compounds.

Computer-assisted drug discovery is also a valuable component complementing our screens for biological activity. Starting from the validated screen, the chemistry team can match the molecular features of hit compounds to large collections of commercially available
compounds and search them for assay (hit-expansion). Other scaffolds for this virtual screening approach include known compounds or crystallographic coordinates. This would yield a small number of compounds of each chemical family for further characterization in terms of biological activity. Comparison between active and inactive analogues will provide useful information for the establishment of a structure-activity relationship. With the expected results, successive design, chemical synthesis and experimental validation cycles are expected to produce compounds with enhanced activities.

The identification of these compounds as new potential inhibitors of necroptosis, together with elucidation of their mechanism of action, warrants further exploration to clarify their potential necroptosis-related clinical application.


45. Brüne B. The intimate relation between nitric oxide and superoxide in apoptosis and cell survival, Antioxidants and Redox Signaling. 2005;7(3-4):497–507.


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Appendix
Supplementary Figure 1 (larger view of Figure 4) - OCA, UDCA, TUDCA as well as SB16 and SB22 inhibit TNF-α-induced L929 cell necroptosis. L929 cells were incubated with 30 µM TNF-α in the presence or absence of either 30 µM Nec-1 or 30, 60 or 100 µM BAs and derivatives. Necroptosis was determined using the ToxiLight™ BioAssay Kit. Results are presented as the mean value ± SEM for at least three independent experiments performed in duplicates. N/A, no addition. §p < 0.05 and *p < 0.01 vs. N/A; †p < 0.05 and ‡p < 0.01 vs. TNF-α.
Supplementary Table 1 – Chemical and structural characteristics of OXAs

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