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REVIEW

Pharmacological modulation of cell death in organ transplantation

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SUMMARY

New options to pharmacologically modulate fundamental mechanisms of regulated cell death are rapidly evolving and found first clinical applications in cancer therapy. Here, we present an overview on how the recent advances in the understanding of the biology and pharmacology of cell death might influence research and clinical practice in solid organ transplantation. Of particular interest are the novel opportunities related to organ preservation and immunomodulation, which might contribute to promote organ repair and to develop more selective ways to modulate allogeneic immune responses to prevent rejection and induce immunological tolerance.

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Introduction

Fifty years after the introduction of the concept of *programmed cell death* and thirty years after the identification and cloning of B-cell lymphoma 2 Bcl2 as the first molecular component of a regulated cell death mechanism, these discoveries are translating into novel therapeutic approaches [1,2]. Drugs rationally designed to selectively modulate cell survival and cell death have been successfully tested in patients with hematological malignancies, thereby defining a new class of pharmacological targets [3]. It is not surprising that the most advanced clinical studies in this emerging field were performed in oncology, as cell survival dysregulation is a hallmark of cancer and anti-apoptotic factors such as Bcl2 are an important class of oncogenes. However, in consideration of the fundamental role of cell death regulation in a variety of biological processes (such as in development, tissue repair, or immune regulation) and in the pathophysiology of many diseases (such as autoimmunity or neurodegenerative disorders), it is likely that cell death pathways will encompass pharmacological targets with clinical relevance beyond cancer therapy. Here, we discuss how recent advances in the understanding of cell death biology and the development of drugs selectively modulating cell death might influence research and clinical practice in solid organ transplantation.

Recent advances in the understanding of regulated cell death

Several modes of cell death have been described and characterized in the last decades: Well-known processes, such as necrosis or apoptosis, were accompanied by other cellular ways to die, including among others necroptosis, netosis, ferroptosis, oxytosis, or entosis [4]. The classical morphological classification in apoptosis (type I cell death), autophagy (type II cell death), and necrosis (type III cell death) is currently considered an

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oversimplification of a multifaceted biological process [5]. According to the recommendations of the Nomenclature Committee on Cell Death (NCCS), the classification of the different modes of cell death should be based on quantifiable biochemical parameters and functional considerations [6]: For example, "extrinsic apoptosis by death receptor" is defined by death receptor signaling and caspase 8 activation, whereas entosis is defined by Rho and Rock1 activation. This approach should provide a precise and comparable definition of cell death and facilitate communication among scientists, but it is not very understandable for people not working on a daily basis in this rapidly evolving field [6]. Therefore, a more practical, operational approach was recently proposed to help the nonexpert to understand the principles of cell death biology and to discriminate "essential and accessory aspects of cell death" [5]. This approach is supported by recent studies suggesting that, despite the complexity of cell death regulation, the fundamental execution mechanisms are probably more homogeneous than previously thought [7].

According to the NCCS [5], a first broad distinction between *accidental* and *regulated* cell death should be made. Accidental cell death results from a severe damage of the cell (such as by mechanical or thermal injury) and is immediate, unpredictable, and not modifiable by pharmacologic interventions. In contrast, *regulated cell death* (RCD) involves genetically encoded mechanisms and can be modified pharmacologically. RCD can occur as a result of an external trigger, but also in physiological conditions, such as in the context of development or immune regulation (in this case usually referred to as *programmed cell death*) [5]. Independently of the exact mode of cell death, the process of RCD can be divided into an initiation, execution, and propagation phase (Fig. 1). The initiation phase is triggered by a homeostatic perturbation leading to a cellular stress response. In this phase, the integration of different cell intrinsic and extrinsic signals will determine whether the perturbing stimulus can be overcome and the cell/tissue repaired, or whether the execution phase will be started. In contrast to the initiation phase, the execution phase is per definition irreversible. The point of no return is usually defined by the activation of executioner mechanisms, such as the activation of caspase 8 or caspase 9. Importantly, the functional relevance of RCD does not cease with the death of the single cell. In the propagation phase, dying cells release a multitude of mediators (damage-associated molecular patterns – DAMPs) that influence neighboring cells and tissue homeostasis: For example, RCD can induce a second wave of cell death independently of the initial stimulus (e.g., in the process of apoptosis-induced apoptosis [8]), stimulate cell proliferation and tissue repair [9], or trigger inflammatory responses [10]. Thus, RCD is relevant beyond the definition of the fate of a single cell; it orchestrates fundamental processes of tissue homeostasis at the local and systemic level. The impact of these processes has been recognized in different conditions including cancer [10] and autoimmunity [11]. Here, we provide a general perspective on the role of RCD in solid organ transplantation with a particular interest in the identification of therapeutic targets to pharmaceutically exploit these mechanisms.

Pharmacological modulation of cell death

The growing knowledge of the molecular mechanisms of RCD culminated in the development of new approaches to modulate life-and-death decisions within a cell. Apoptosis has been a main interest of drug

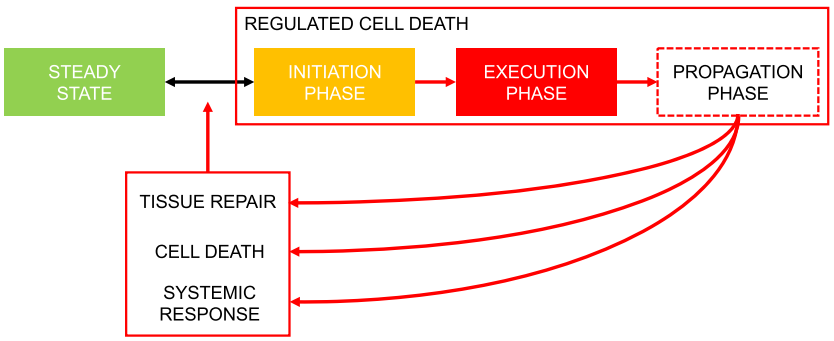

Figure 1 General process of regulated cell death. Regulated cell death … Cell death occurs in subsequent phases independently of the exact molecular mechanisms. From its steady state, a cell enters the process of regulated cell death through an initiation phase, leading to an execution phase and culminating in the propagation phase. The propagation phase will influence fundamental biological processes at the local and systemic level and might be of particular relevance in organ transplantation.

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discovery for many years, and several apoptosis modulators have been tested in clinical trials in oncology. We will primarily focus on apoptosis modulators, as these drugs are more likely to be available for (off-label) clinical applications soon (Fig. 2), but other forms of RCD might emerge as potential therapeutic targets in transplantation medicine in the future [12–14].

A first generation of pharmaceuticals were designed to trigger the extrinsic apoptosis pathway by binding the death receptors, including CD95/Fas, TRAIL-R, and TNF [15]. Some of these compounds, such as the monoclonal antibody agonist to TRAIL-R1 mapatumumab, displayed a favorable toxicity profile and were tested in clinical trials in patients with cancer [16]. Anti-CD95/Fas antibodies were investigated also in the field of transplantation with the purpose to selectively induce apoptosis in alloreactive T cells, but this strategy had to be abandoned because of severe hepatic side effects [17,18]. Caspases and inhibitors of apoptosis (IAPs) were also recognized as potential pharmacological targets. Spinal cord, myocardial, and liver injury were beneficially influenced by caspase inhibitors in different experimental models, including ischemia/reperfusion injury [19,20]. Moreover, blocking caspases had an anti-inflammatory effect and improved survival in septic mice [21]. Different approaches were developed to inhibit IAPs, as this family of caspase-inhibiting proteins was recognized as a pivotal checkpoint of apoptosis regulation [22,23]. As an example, antisense targeting of Xiap (X-linked inhibitor of apoptosis protein) sensitized tumor cells to radio- or chemotherapy. The caspase activator Smac (second mitochondria-derived activator of caspases) inhibits Xiap, and the refinement of the pharmacological properties of Smac mimetics might lead to novel compounds of interest [13].

Among the several potential pharmacological targets to modulate apoptosis, the Bcl2 family is the best investigated and currently the most relevant in a clinical perspective [24]. The interaction of pro- and anti-apoptotic factors of the Bcl2 family controls the permeabilization of the outer mitochondrial membrane and therefore the release into the cytoplasm of cytochrome c, Smac, and other factors involved in the formation of the apoptosome, a large protein complex, which activates pro-caspase 9. Bcl2 factors can be divided into three groups according to their molecular structure and function [13]. The pro-apoptotic factors Bax and Bak are directly involved in the process of pore formation in the mitochondrial membrane. In steady state conditions, this process is inhibited by different anti-apoptotic factors

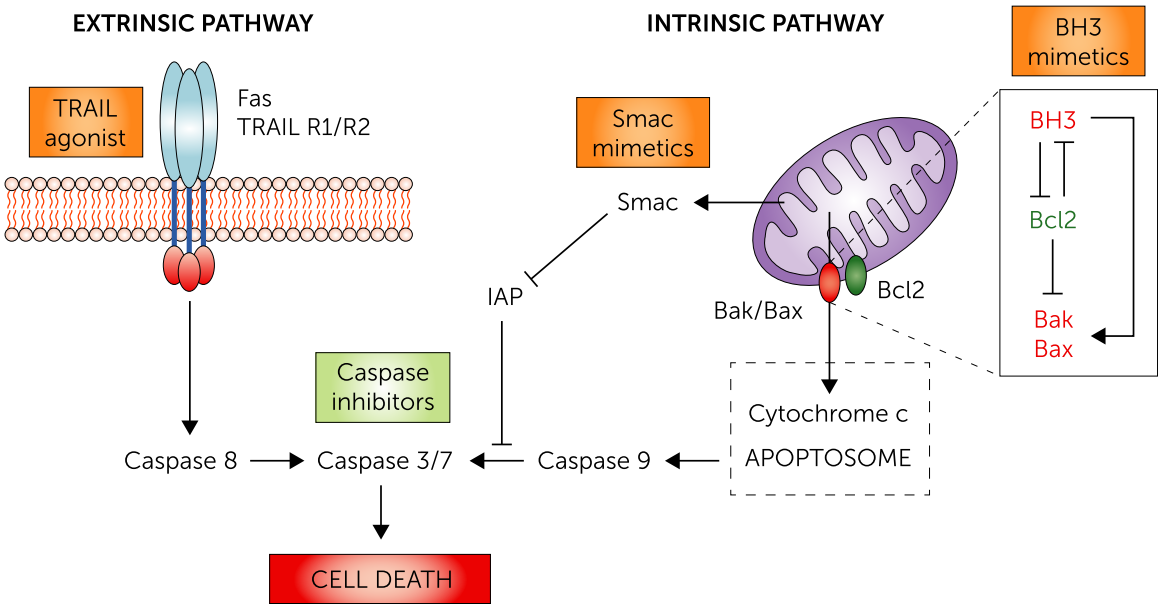

Figure 2 Pharmacological apoptosis modulation. Simplified overview of the apoptosis pathways and of the currently most relevant approaches for pharmacological modulation. Apoptosis is mediated by two interconnected pathways. The extrinsic pathway is triggered by membrane receptors and leads to the activation of pro-caspase 8. The intrinsic (or mitochondrial) pathway regulates the permeabilization of the outer mitochondrial membrane and activates pro-caspase 9. Both pathways converge to a common execution pathway that mediates cell death. The complex interaction between the pro-apoptotic (in red) and anti-apoptotic (in green) factors of the Bcl2 family is presented on the upper right side. The most important classes of drugs selectively modulating these processes are indicated in the full boxes (red for pro-apoptotic and green for anti-apoptotic drugs).

(such as Bcl2, Bclxl, or Mcl1) that mutually interact with Bax/Bak and BH3-only proteins. This latter group of pro-apoptotic factors, including among others Bim, Bid, and Puma, can activate the apoptosis cascade by direct activation of Bax/Bak or indirectly by inhibiting the anti-apoptotic Bcl2 factors [25,26] (Fig. 2). Similarly, anti-apoptotic Bcl2 factors can be inhibited pharmacologically with peptides, antisense oligonucleotides, or small molecules [13]. The characterization of the first small-molecule BH3 mimetic, ABT-737, was published in 2005 [27]. As the survival of different cells depends on the expression of different pro- and anti-apoptotic factors, the binding selectivity of each compound determines its efficacy and toxicity [28]. ABT-737 and its orally bioavailable counterpart (ABT-263 or navitoclax) bound Bcl2, BclxL, and Bclw and efficiently induced apoptosis in cancer cells, but also caused thrombocytopenia (as platelet life span depends on BclxL [29]). To overcome this dose-limiting side effect, ABT-263 was reengineered to develop ABT-199 (venetoclax), a selective Bcl2 inhibitor [30]. This compound was successfully investigated in patients with relapsed chronic lymphocytic leukemia and was recently approved by the FDA [3,31]. ABT-263 and ABT-199 are currently being tested in patients with other solid and hematological cancers. Furthermore, in consideration of the immunomodulatory properties of these drugs, explained in more detail below, clinical trials with ABT-199 are currently ongoing in patients with systemic lupus erythematosus [32,33]. The pharmacological inhibition of Mcl1 was more challenging, but the recent development of a selective small-molecule Mcl1 inhibitor provides a new option to deplete plasma cells, an opportunity with clinical applications beyond the therapy of plasma cell dyscrasias, for example, for the prevention and treatment of humoral rejection [34,35].

An area of major potential clinical relevance is the pharmacological modulation of regulated necrosis. Necroptosis and ferroptosis are involved in several clinically relevant conditions including ischemia/reperfusion injury and are linked to the regulation of the inflammatory response [36–38]. The relevance of these processes in transplantation has been demonstrated in several experimental models [39,40]. Selective modulators of regulated necrosis are currently not available for clinical applications, but major advances were achieved in the last years [14]. Moreover, several drugs commonly prescribed to transplant recipients (such as mTOR inhibitors or statins) might influence necroptosis or other forms of RCD as an "off-target" effect of potential functional relevance [41,42].

Thus, several molecular targets to modulate cell death have been identified in the last years, and the development of new highly selective drugs opens the opportunity to precisely target these mechanisms in different clinical conditions. The recent approval of the first BH3 mimetic by the FDA will accelerate the clinical use of this new class of drugs also beyond the primary indication, and this approach might find an application also in organ transplantation. Here, we propose two potential applications of clinical relevance in organ transplantation: tissue repair and immune regulation.

Allograft biology: from preservation to repair

The detrimental effects of cell death in organ transplantation have been extensively characterized and promulgated over the years. According to the dogma, preventing cell death is crucial to preserve organ function. In fact, apoptosis was identified as a mechanism of organ injury in liver transplantation and – as mentioned above – caspase inhibitors were experimentally used to protect liver allografts from ischemia/reperfusion injury [19,43]. Moreover, apoptosis and necroptosis contributed to acute kidney injury in the early post-transplant period and reduced renal allograft survival [39]: That is, the number of apoptotic cells in kidney biopsies obtained before implantation was associated with delayed graft function and allograft dysfunction in the first months after transplantation (but interestingly not in the long term) [44]. Analogous observations were reported in heart, lung, and pancreas transplantation [45–47]. However, the link between the different forms of RCD and allograft function might be more variegated: Autophagy displayed a dual role in renal ischemia/reperfusion injury and in kidney transplantation with beneficial or deleterious effects depending on the experimental model [48,49]. Blocking caspase 8 ameliorated renal ischemia/reperfusion injury but resulted in a reduced allograft survival, because of accelerated necroptosis [39]. These observations indicate that preventing cell death by any means might not be a clever approach and that the molecular crosstalk between different forms of cell death needs to be considered to obtain the best organs in the long term [50].

More recently, RCD was recognized as a fundamental step in the cell biology of tissue repair [51–53]. In models of tissue regeneration in *Hydra*, apoptosis is necessary to induce proliferation of surviving cells and modulate regeneration [54], and similar processes have been described in different models of wound healing in several species [55,56]. More precisely, both initiator

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and executioner caspases mediate the activation of fundamental pathways, such as Wnt or Hedgehog, which induce compensatory cell proliferation and tissue repair [52]. Similarly, receptor-interacting protein kinase 3 (Ripk3), a pivotal element of necroptosis regulation, was not only involved in the inflammatory response after injury, but also in injury-induced tissue repair in a colitis model [57].

These considerations might be particularly relevant in the current era of organ transplantation. To improve long-term graft function despite organ scarcity, with the need to include marginal donor to enlarge the donor pool, it is crucial to exploit the complete potential of the transplanted tissues [58]. In this context, organ preservation might not be sufficient and supporting mechanisms of tissue repair might be required, as supported by recent data linking repetitive injury episodes and the expression of genes involved in renal development and repair with allograft fibrosis [59]. Accordingly, studies including kidney donors with severe acute kidney injury indicated that cell death-associated genes were upregulated in recipients of an injured kidney, but the long-term outcome as assessed by renal function, histology, and gene expression profile did not differ compared to recipients of a noninjured graft [60]. Notably, RCD might be crucial not only in the acute phase after transplantation, but also in the pathological processes leading to chronic graft dysfunction and fibrosis [61]. Anoikis, that is apoptosis induced by a disturbed interaction between the cell and the extracellular matrix, is involved in the remodeling of the extracellular matrix and might be of particular relevance in this context [62,63].

In a clinical perspective, it is important to recognize that the advent of novel techniques for organ perfusion offers the opportunity not only to preserve but even to enhance organ quality by *ex vivo* manipulation [64–66]. A pharmacological manipulation of RCD, not necessarily aiming at inhibiting cell death, but rationally targeting mechanisms of tissue repair, might be an interesting approach in this context.

Targeting cell death for immunomodulation

The inflammatory response induced by ischemia/reperfusion injury is a critical element in the activation of the allogeneic immune response in transplant recipients. Necroptosis is currently considered the critical link between tissue damage and inflammation, thereby defining potential new therapeutic targets to prevent rejection [36,39,57].

The role of apoptosis as an executioner and a regulator of the immune system is well established. Fundamental processes in the development and the regulation of the adaptive immune system are mediated by apoptosis [67,68]: Positive and negative selection of lymphocytes in central lymphatic organs and the T-cell contraction phase after antigen clearance are important examples in this regard. The fate of each single lymphocyte is controlled by a very dynamic modulation of the apoptosis pathway, as determined by the developmental and metabolic state of the cell and by a variety of extrinsic stimuli [69,70]. As an example, survival of naïve T cells depends on Bcl2, which counteracts the pro-apoptotic factor Bim [71]. Bcl2 expression depends on IL-7 and on a basal calcineurin activity [72–74]. This delicate balance dramatically changes after antigen recognition: T-cell activation triggers the expression of anti-apoptotic factors (such as c-Flip, Bcl2a1, and Xiap), but also of pro-apoptotic factors (such as TRAIL-R1 and Bim) [75–78]. Under the control of costimulatory molecules and cytokines, the balance between pro- and anti-apoptotic factors dictates whether a cell is supposed to survive or to die [76]. Thus, lymphocytes live in perpetual selection process, a state similar to the initiation phase of RCD (Fig. 1), prone to undergo cell death as soon as the required pro-survival stimuli are downregulated. In consideration of the potential harmful effect of an uncontrolled activation of immune cells, the continuous selection of lymphocytes is considered a fundamental principle of immune regulation and apoptosis is instrumental in this indispensable mechanism to prevent autoimmunity [67].

For the precise regulation of lymphocyte selection, the immune system makes use of the whole complexity of the apoptosis pathway. Different pro- and anti-apoptotic factors are expressed among various lymphocyte subpopulations and are regulated by different stimuli. Focusing only on the Bcl2 family, several studies revealed that Bcl2 is the critical anti-apoptotic factor in naïve and memory lymphocytes [71,79], Bcl2a1 is important in activated T cells [78,80], and Mcl-1 is pivotal for the survival of regulatory T cells [81] and plasma cells [35]. Interestingly, the distinctive expression of Bcl2 family factors at different stages of lymphocyte differentiation and in different lymphocytes subsets offers the opportunity to selectively induce apoptosis in defined cell subsets and therefore to enrich or deplete determined cells populations using specific BH3 mimetics. We made use of this approach to deplete donor-reactive T cells in combination with costimulation

blockade and to enrich regulatory T cells using ABT-737 [77,82].

BH3 mimetics might therefore assume different immunomodulatory properties. ABT-737 displayed a favorable effect in models of autoimmunity, including lupus, rheumatoid arthritis, and inflammatory bowel disease [32,83]. As mentioned above, the beneficial properties of ABT-199 in systemic lupus erythematosus are currently being investigated in clinical trials [33]. ABT-737 inhibits allogeneic immune responses by a multifactorial mechanism of action including a general lymphopenia, a selective deletion of donor-reactive T cells in combination with costimulation blockade and by regulatory T-cell enrichment [77,82,84,85]. This effect was markedly increased in combination with cyclosporine A, as calcineurin inhibitors also substantially influence apoptosis regulation in lymphocytes [74,78,86]. The same approach can also be used to target memory cells, and the advent of selective Mcl1 inhibitors might offer the opportunity to deplete plasma cells, a lymphocyte population relatively resistant to currently available immunosuppressive drugs [35,87]. Furthermore, in line with the well-characterized critical role of apoptosis in the maintenance of immune tolerance [67,88], ABT-737 displayed a multifactorial tolerogenic effect and a short induction therapy with ABT-737, cyclosporine A, and costimulation blockade was sufficient to induce stable mixed lympho-hematopoietic chimerism and long-term immunological tolerance across MHC barriers without myelosuppressive therapy [77]. Thus, drugs selectively targeting cell death pathways can be considered as a novel class of immunomodulatory drugs with great potential in the field of organ transplantation.

A pragmatic approach toward novel therapeutic options

Regulated cell death is a fundamental biological process. In this review article, we introduced two possible applications of drugs modulating RCD in solid organ transplantation: tissue preservation/repair and immunomodulation. However, the use of these compounds in a multifactorial clinical scenario such as organ transplantation might be very challenging. The different modes of cell death are interconnected, and their regulation and systemic effects might change depending on the organ of interest and the experimental model [89,90]. Moreover, in a particular clinical setting, it may be desirable to induce cell death in a well-defined cell population and protect other cells from cell death simultaneously: That is, it might be attractive to induce apoptosis in plasma cells producing donor-reactive antibodies and – at the same time – to protect kidney tubular cells to preserve renal function. Moving from a conceptual to a pragmatic approach, the central question is: Is it possible to dissect the deleterious and beneficial effects of RCD to exploit the potential of this approach in organ transplantation?

In our opinion, three principles need to be considered to achieve this aim (Table 1). First, to obtain a specific effect it is important to target the right cell. As recently pointed out by R. Lockshin, "each cell has a distinct history and metabolism, so that each cell responds differently in time and response to the same stimulus" [1]. Therefore, different cell populations might substantially differ in the regulation of cell survival, thereby offering the opportunity to achieve selectivity by modulating specific molecular targets [74,84]. As a result, different organs and tissues react in a different way to the inhibition of specific Bcl2 factors and the selectivity profile of each BH3 mimetics determines its toxicity [67,79]. Even the same cell type can react differently to a pro-apoptotic stimulus depending on the environment and its metabolic state [78]. Thus, a detailed understanding of apoptosis regulation in different organs/tissues/cells combined with the application of drugs selectively targeting key regulators of apoptosis with high molecular selectivity will be important to achieve cell specificity *in vivo* and *ex vivo*. The application of new technologies, such as single-cell RNA sequencing, will contribute to the understanding of cell-to-cell heterogeneity at a much higher resolution [91].

Second, different modes of cell death can result in opposite responses. Therefore, it is important to carefully select which cell death pathway should be inhibited or stimulated in a defined clinical condition, and in determined conditions, it might be necessary to simultaneously target different modes of cell death [14]. The classical sharp differentiation in immunogenic and tolerogenic cell death is an oversimplification of a complex process, and the same mode of RCD can induce different responses, as recently reviewed for apoptosis in the context of transplantation [11,89]. However,

Table 1. Principles for a pragmatic application of cell death modulators in experimental models of organ transplantation.

1. Target the right cell population
2. Target the right cell death pathway
3. Consider the propagation phase of cell death

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different modes of RCD trigger the immune system in a different way [11]. The relevance of these issues is increasingly recognized in cancer immunology [10,92], and it is likely that future studies will provide important information to create the optimal conditions for a *tolerogenic* and *regenerative* cell death.

Third, in a complex biological system such as in solid organ transplantation, the propagation phase of cell death with its local and systemic effects is probably crucial and needs to be considered in appropriate experimental models (Fig. 1). Thus, focusing on RCD in the single cell is probably not appropriate to develop new therapeutic strategies in transplantation. *In vitro* experiments with cancer cell lines substantially contributed to the development of drugs targeting RCD, but are not appropriate to understand the mechanism of action of a particular drug in a transplant recipient, and unexpected off-target effects, including interactions with immunosuppressive drugs [74], should always be taken into account [82]. The first experience with BH3 mimetics revealed that this principle is probably applicable also in cancer therapy [93].

In conclusion, the approval of ABT-199 by the FDA introduced a new class of drugs to selectively modulate fundamental biological processes regulating cell survival and cell death. The potential applications of these compounds can be extended beyond cancer therapy and might be of interest in the field of organ transplantation. This will stimulate the transplant community to reconsider the old dogma of RCD in the context of tissue preservation/repair. Moreover, this new class of immunomodulatory drugs might be useful to deplete immune cells refractory to currently available therapies, and to induce immunological tolerance.

Authorship

PEC and TF: wrote the manuscript.

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