Teaser This review provides an up-to-date overview of the different peptides that have emerged as modulators of ER–mitochondrial Ca^{2+} fluxes with translational and therapeutic potential.

Therapeutic implications of novel peptides targeting ER–mitochondria Ca^{2+}-flux systems

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Intracellular Ca^{2+}-flux systems located at the ER–mitochondrial axis govern mitochondrial Ca^{2+} balance and cell fate. Multiple yet incurable pathologies are characterized by insufficient or excessive Ca^{2+} fluxes toward the mitochondria, in turn leading to aberrant cell life or death dynamics. The discovery and ongoing molecular characterization of the main interorganellar Ca^{2+} gateways have resulted in a novel class of peptide tools able to regulate relevant protein–protein interactions (PPIs) underlying this signaling scenario. Here, we review peptides, molecularly derived from Ca^{2+}-flux systems or their accessory proteins. We discuss how they alter Ca^{2+}-signaling protein complexes and modulate cell survival in light of their forthcoming therapeutic applications.

Introduction

In vertebrates, Ca^{2+} outside bones and teeth amounts only to <1% of the total Ca^{2+}. However, this Ca^{2+} is used intracellularly to drive various physiological processes, from fertilization, gene transcription, exocytosis and cell cycle progression to cell death [1–4]. The spatiotemporal profile of these Ca^{2+} signals and their decoding by Ca^{2+}-binding proteins are key to this whole spectrum of functions. As such, the free cytosolic Ca^{2+} concentrations are kept very low at ~100 nM. This is achieved by several Ca^{2+}-transport systems that extrude Ca^{2+} from the cytosol, including Na^+/Ca^{2+} exchangers, plasma membrane Ca^{2+} ATPases, sarco/endoplasmic reticulum (ER) Ca^{2+} ATPases (SERCA) and Ca^{2+}-binding proteins. Inside the cell, the majority of the Ca^{2+} is stored in the ER [$(Ca^{2+})_{ER}$ ~500μM], although other compartments also store Ca^{2+}, such as the Golgi and the lysosomes, whereas the mitochondria can transiently accumulate Ca^{2+}.

Mitochondrial Ca^{2+} accumulation is crucial for several mitochondrial functions, and by extension cell survival and cell death (Fig. 1 provides a simplified overview). The Krebs cycle, for example, is dependent on Ca^{2+} by virtue of its binding to and activation of enzymes such as pyruvate, citrate and α-ketoglutarate dehydrogenase, driving ATP output. In the absence of such Ca^{2+} fluxes, ATP output diminishes, resulting in the activation of AMP-activated kinases and

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Martijn Kerkhofs started his PhD in the LMCS research group, KU Leuven (Belgium), in 2016. The main aim of his research work is to exploit the Bcl-2-IP_3R interaction in diffuse large B cell lymphomas using IP_3R-derived peptide tools to drive cell death in Bcl-2-dependent cancer cells and elucidating the underlying molecular and cellular mechanisms.

Geert Bultynck is a professor in the Laboratory of Molecular and Cellular Signaling (LMCS) in the Department of Cellular & Molecular Medicine, KU Leuven (Belgium), since 2008 onwards. His research aspires to explore and to exploit the role of intracellular Ca^{2+}-release channels and their modulation accessory proteins such as Bcl-2 proteins in cell death and survival in health, disease and therapy. The aim is to develop novel therapeutic strategies to tackle diseases linked with altered activity of IP_3 and ryanodine receptors.

Tim Vervliet obtained his PhD in biomedical sciences in 2015. He is a post-doctoral researcher at KU Leuven (Belgium) in the LMCS research group. His research focuses on elucidating how endoplasmic reticulum-located ryanodine receptor Ca^{2+}-release channels are regulated by Bcl-2 proteins in health and disease. The aim is to apply these insights and identify peptide tools to target neurodegenerative diseases, myopathies and acute pancreatitis.

Giovanni Monaco is a senior post-doctoral researcher in the LMCS Lab, who received his PhD in biomedical sciences, KU Leuven (Belgium), in 2013. His research interests range from the cellular study of ER˜mitochondria Ca^{2+}-crosstalk and apoptosis signaling (e.g., Bcl-2 family member function) to the rational design of targeted therapies and peptide tools, exploiting the molecular and metabolic vulnerabilities of leukemia and related hematological diseases.

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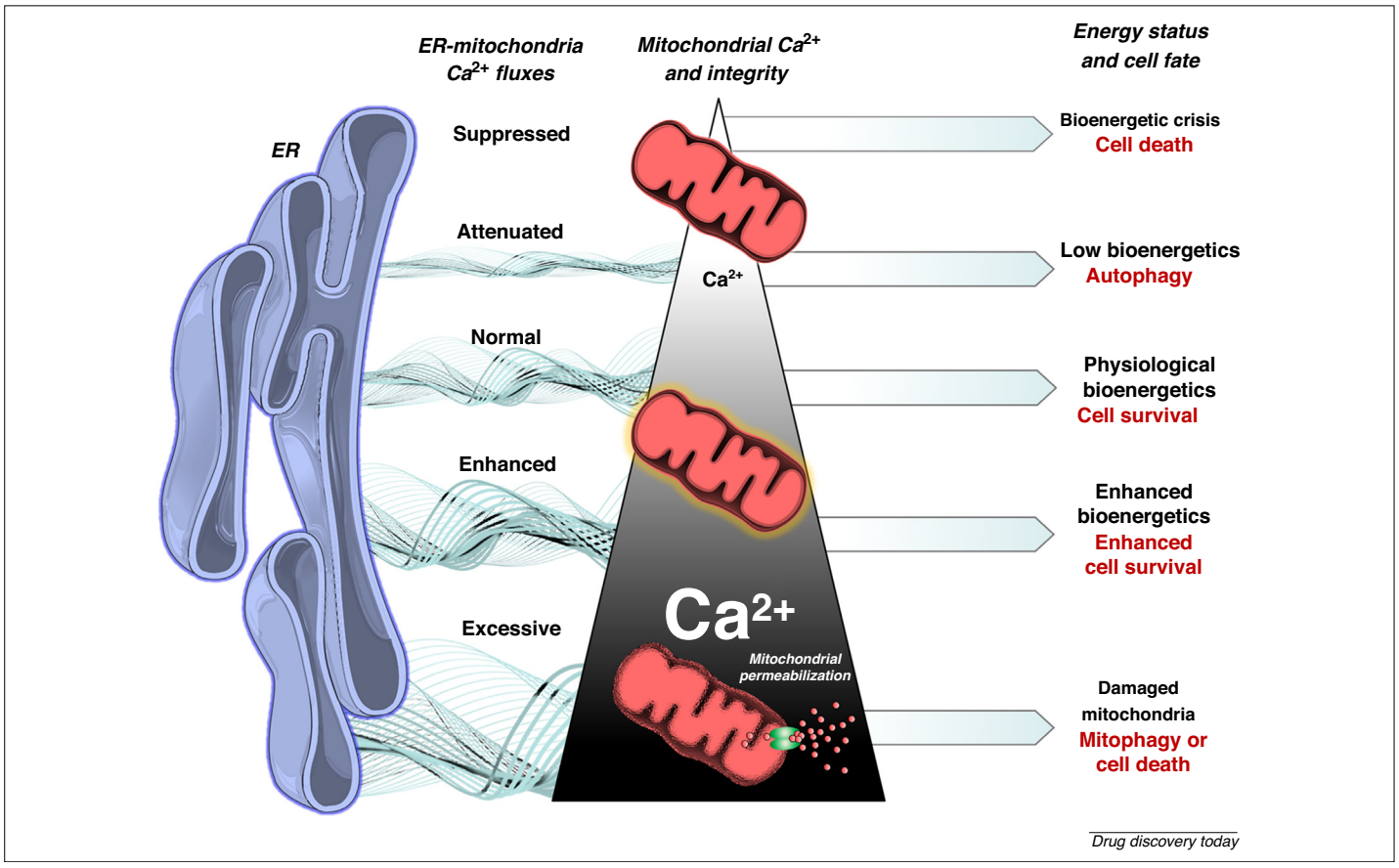

FIGURE 1

Endoplasmic reticulum (ER)–mitochondrial Ca^{2+} exchange regulates cell death and survival, as well as the energy status of the cell. Different levels of Ca^{2+} signaling impact the cell in a different way. Mitochondrial Ca^{2+} uptake directly stimulates mitochondrial energy production. Hence, lack of mitochondrial Ca^{2+} causes a bioenergetic crisis, possibly leading to cell death. If the cell experiences suboptimal Ca^{2+} exchange between ER and mitochondria, autophagy can be activated to supplement the low bioenergetic state. This can lead to physiological bioenergetics and cell survival; however, if the autophagic response is not sufficient to rescue the cells, a bioenergetic crisis could yet ensue. By contrast, enhanced ER–mitochondrial Ca^{2+} flux and subsequent enhanced bioenergetics make cells more resilient and thus enhance cell survival. Lastly, Ca^{2+} overload in the mitochondria can underlie mitochondrial damage. This can cause cell death but in some cases it is a trigger for mitophagy, a mitochondria-specific form of autophagy, to clear defective mitochondria, allowing the cell to survive.

subsequent energy re-supplementation via the cytosolic recycling process of autophagy [5]. Conversely, an excess of Ca^{2+} in the mitochondria, often in combination with the production of reactive oxygen species (ROS) opens the mitochondrial permeability transition pore (mPTP), which renders the inner mitochondrial membrane permeable for all kinds of solutes and hampers mitochondrial function [5,6]. Dysfunctional mitochondria are removed by mitophagy, the selective degradation of mitochondria by autophagy, to prevent cell damage and death [6,7]. Mitochondrial Ca^{2+} accumulation happens through 'quasi-synaptic' Ca^{2+} fluxes between the ER and the mitochondria [8,9]. Given the importance of mitochondria in the regulation of cell fate, an adequate functioning of the ER–mitochondrial Ca^{2+}-signaling axis is important for cell survival. Many parameters impact the extent of ER–mitochondrial Ca^{2+} fluxes. These factors include: expression, properties and regulation of Ca^{2+}-release channels and pumps at the ER [10,11], expression and regulation of mitochondrial Ca^{2+}-uptake proteins [12,13] and the highly negative mitochondrial membrane potential ($\Delta\Psi m$), approximately −180 mV [5] (the main molecular players involved in this Ca^{2+} communication are illustrated in Fig. 2).

To enable ER–mitochondrial Ca^{2+} fluxes, the ER lumen needs to maintain sufficiently high Ca^{2+} levels, which are sustained by SERCA activity and ER luminal Ca^{2+}-binding proteins [14]. Furthermore, two important channel families are responsible for Ca^{2+} release from the ER: the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) [15], which is expressed in most cell types, and the ryanodine receptor (RyR) [16], which has a more distinct expression pattern, for example cardiomyocytes, skeletal muscle fibers, neurons, liver and pancreas. The IP_3R is gated by IP_3, which is formed from phosphatidyl inositol 4,5-bisphosphate (PIP_2) in response to activation of G-protein-coupled receptors (GPCRs) or tyrosine kinases [15]. RyRs are activated in response to a variety of physiological agonists, including Ca^{2+} itself, cyclic-ADP ribose and NAADP or via coupling with dihydropyridine receptors (DHPRs) in skeletal muscles [16]. Furthermore, IP_3Rs and RyRs are tetrameric channels encoded by three distinct genes. They give rise to the expression of three different isoforms each (IP_3R1, IP_3R2 and IP_3R3, and RyR1, RyR2 and RyR3, respectively), which are differentially activated and regulated [10,17]. Upon activation of these channels, Ca^{2+} is released from the ER into the cytosol [15,16]. Mitochondrial Ca^{2+} uptake is mediated by two main channels: the

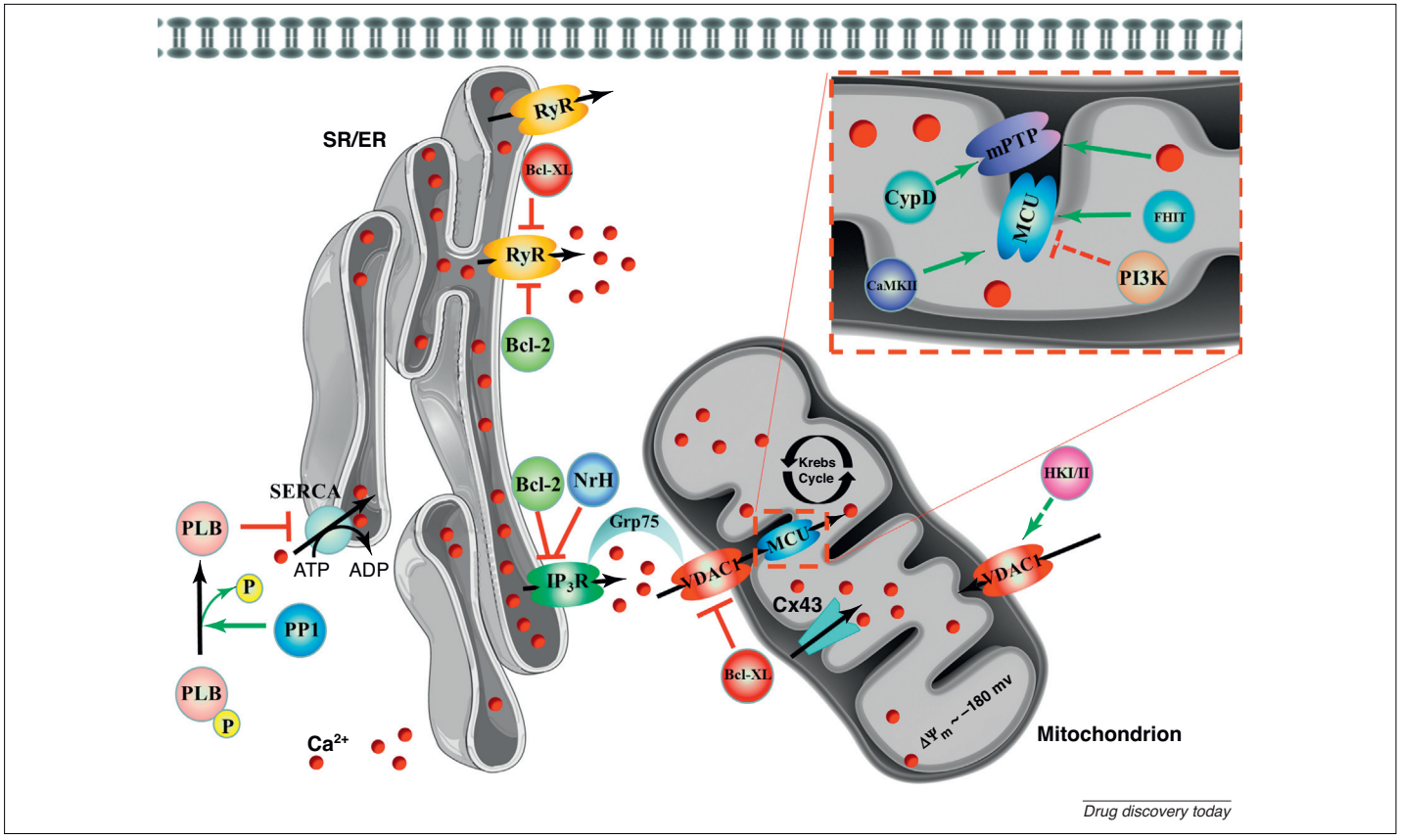

FIGURE 2

Overview of the ER–mitochondria Ca^{2+}-signaling toolkit and its regulators. The ER harbors two main Ca^{2+}-release channels: IP_3R and the RyR. Both Ca^{2+}-release channels are regulated by members of the Bcl-2 protein family, namely Bcl-2 and Nrh for the IP_3R and Bcl-2 and Bcl-XL for the RyR. To retain its filling state, the ER also harbors the SERCA. SERCA activity is controlled by PLB, in itself subject to dephosphorylation by PP1. This dephosphorylation manages PLB activity, enabling increased inhibition of SERCA. On the mitochondrial side, Ca^{2+} traverses the OMM via VDAC1. VDAC1 conductivity can be modified by interacting proteins such as HKI/II or Bcl-XL. After crossing the OMM, Ca^{2+} is transported to the matrix via the MCU. MCU itself is regulated through phosphorylation, for example by CaMKII, but sensitization also occurs through an interaction with FHIT. Additionally, PI3K indirectly inhibits MCU activity. In the IMM, the mPTP is present too. This hitherto unidentified channel permeates the IMM in a nonselective way, causing osmotic pressure, mitochondrial swelling and bursting, with subsequent cell death as a result. The mPTP is activated by an increase in mitochondrial Ca^{2+} and its sensitivity toward activation is controlled by CypD. Red lines indicate inhibition, whereas green lines represent stimulation. Dotted lines show an indirect effect. Abbreviations: ER, endoplasmic reticulum; IP_3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor; Bcl, B cell lymphoma; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; PP1, protein phosphatase 1; OMM, outer mitochondrial membrane; VDAC1, voltage-dependent anion channel 1; MCU, mitochondrial Ca^{2+} uniporter; FHIT, fragile histidine triad; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PI3K, phosphoinositide 3-kinase; IMM, inner mitochondrial membrane; mPTP, mitochondrial permeability transition pore; CypD, cyclophilin D.

voltage-dependent anion channel (VDAC) for Ca^{2+} transport across the outer mitochondrial membrane [12] and the mitochondrial Ca^{2+} uniporter (MCU) complex for Ca^{2+} transport across the inner mitochondrial membrane [13]. To prevent an excess of Ca^{2+} accumulation in the mitochondrial matrix, the $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX) exchanges matrix Ca^{2+} for Na^+ or Li^+ in the intermembrane space [18]. Functionally, all these Ca^{2+}-release and Ca^{2+}-uptake proteins can be regulated on many levels, including post-translational modifications and regulation by protein–protein interactions (PPIs), to increase or decrease their Ca^{2+}-release and/or -uptake capacities [12,13,15,16]. The highly negative mitochondrial membrane potential results in a high driving force for Ca^{2+} accumulation in the mitochondria [5].

Another crucial factor that influences ER–mitochondrial Ca^{2+} fluxes is the structure of the micro-domain where this Ca^{2+} exchange takes place [19]. These ER–mitochondrial contact points can be biochemically isolated as mitochondria-associated ER membranes (MAMs), which are ER membranes in close juxtaposition to the mitochondria through physical tethers. In the MAMs, Ca^{2+} concentrations can amount to a multiple of global cytosolic Ca^{2+} concentrations (10 μM range and higher in the MAMs versus 1 μM range in the cytosol), which enables efficient and effective ER–mitochondrial Ca^{2+} signaling. MAM structure is determined by protein tethers and spacers. These proteins regulate the distance between the ER and the mitochondria at the MAMs, which ranges from 15 to 30 nm [20]. Tethers connect the ER to the mitochondria by forming proteinaceous bridges (e.g., IP_3R interacts with VDAC through GRP75 as a molecular bridge [21]), whereas spacers increase the distance at the MAMs. This distance, in part, regulates sensitivity of the mitochondria to ER-mediated Ca^{2+} signals [22]. Similar tethering structures are also observed between mitochondria and the SR, allowing for RyR1-mediated Ca^{2+} signaling toward the mitochondria and proper regulation of excitation–contraction (EC) coupling [23,24].

All these factors add to adequate ER–mitochondrial Ca^{2+} exchange. In pathological conditions, the proper activity of the ER–mitochondrial Ca^{2+}-signaling axis is often disturbed. The malfunction can occur through a direct effect on the Ca^{2+}-signaling machinery, for instance in the case of IP_3R mutations [25]; but in some cases it is not the functionality of the Ca^{2+} channels per se that is disrupted but rather their regulation. For example, different types of cancers are characterized by an overexpression of the antiapoptotic B cell lymphoma-2 (Bcl-2) protein (cfr. infra) [26,27]. Bcl-2, in its canonical function, interacts with and inhibits proapoptotic proteins from the Bcl-2 protein family via its hydrophobic cleft, thereby preventing mitochondrial outer membrane permeabilization (MOMP) and release of proapoptotic factors in the cytosol [28]. Bcl-2 also binds to the IP_3R, thereby suppressing Ca^{2+} signaling from the IP_3R and protecting cancer cells from mitochondrial Ca^{2+} overload and cell death, conferring de facto apoptotic resistance [29]. Another mechanism of regulation involves altered stability of Ca^{2+}-transport systems. For example, the F-box protein L2 (FBXL2), a subunit of the SCF (SKP1-cullin-F-box) ubiquitin protein ligase complex and the tumor suppressor BRCA1-associated protein 1 (BAP1), regulates expression levels of the IP_3R, particularly the type 3 isoform (IP_3R3) [30,31]. FBXL2 binds to IP_3R3, enabling its ubiquitylation and its targeting for proteasomal degradation. However, phosphatase and tensin homolog (PTEN), a tumor suppressor, competes with FBXL2 for IP_3R3 binding, thereby preventing its degradation and proapoptotic Ca^{2+} signaling. However, upon loss of PTEN in cancer cells, a frequently occurring alteration, FBXL2 binding to the IP_3R3 is enhanced, resulting in increased IP_3R3 degradation and resistance against Ca^{2+}-induced cell death [31]. Conversely, the tumor suppressor BAP1, although frequently mutated owing to environmental stresses, binds to and deubiquitylates IP_3R3, stabilizing the channel and sustaining proapoptotic Ca^{2+} signaling in response to cell stress, preventing oncogenesis and malignant cell behavior [30]. Alternatively, MAM organization and/or structure could be altered to contribute to pathological situations. For example, in certain cancers the protein FATE1, which acts as a spacer, is overexpressed. This increases the distance between ER and mitochondria and the MAMs [22]. In turn, this causes reduced sensitivity to ER–mitochondrial Ca^{2+} signaling, and thus mitochondrial Ca^{2+} overload. In this manner, some cancer types gain apoptotic resistance [22].

These examples highlight that at least some pathologies are inflicted because of dysregulated protein interactions. A good approach to alter such protein complexes is the use of peptides [32]. Although the use of peptides poses a number of problems, such as feeble chemical and physical stability, and low plasma half-life, their high efficacy, safety and tolerability provide researchers with a valuable tool to tackle diseases [32]. One of the biggest success stories in terms of peptide drugs is insulin, which was marketed for the first time in 1923 for the successful treatment of

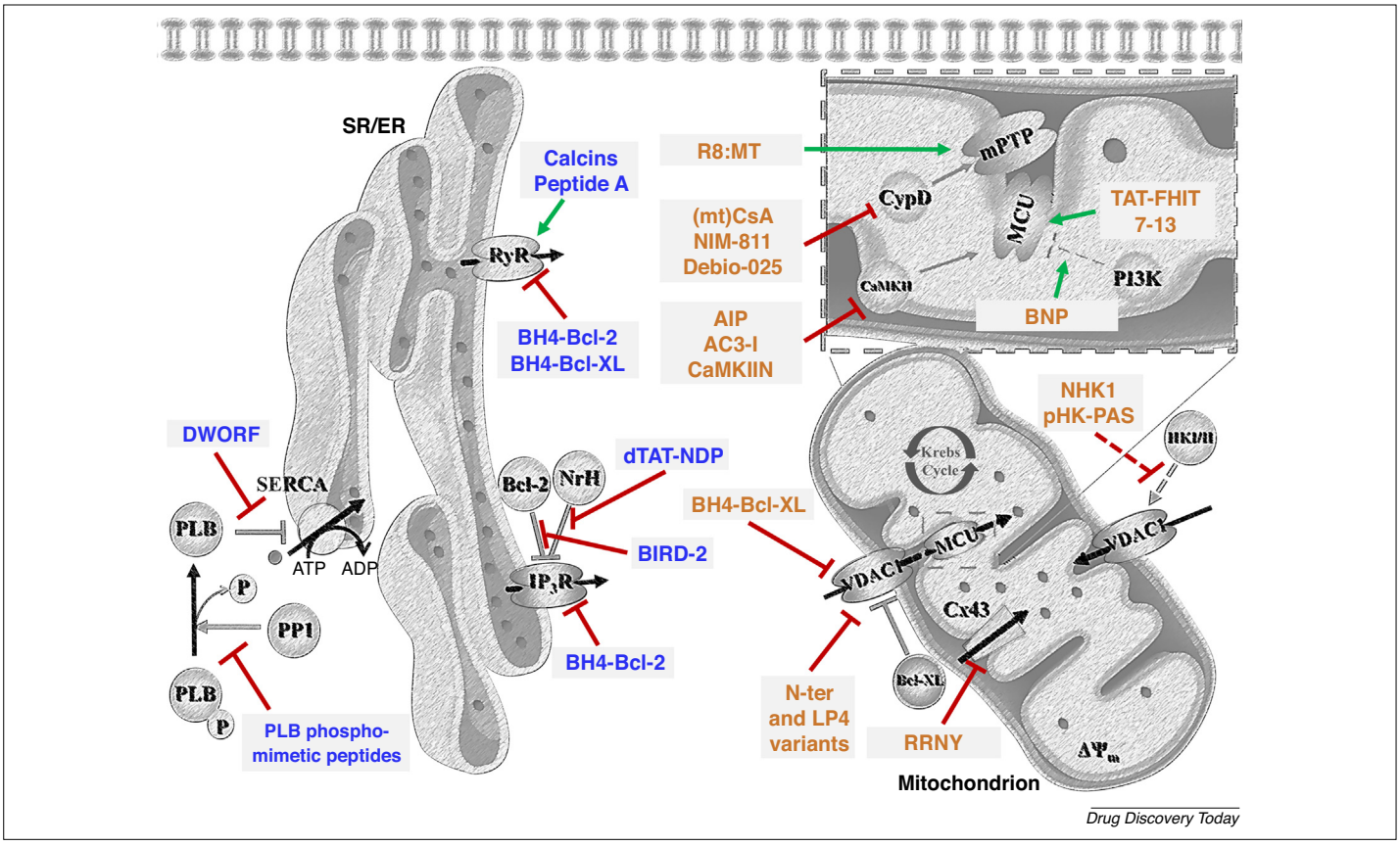

FIGURE 3

Peptides modifying endoplasmic reticulum (ER)–mitochondrial Ca^{2+} fluxes and their targets. Peptides indicated in blue target ER components of the Ca^{2+}-signaling toolkit. Peptides in brown affect mitochondrial proteins responsible for mitochondrial Ca^{2+} signaling. Red lines indicate inhibition, whereas green lines represent stimulation. Dotted lines shown an indirect effect.

diabetes type I, with recombinant insulin entering the markets in 1982 [33]. Currently, peptides are used in medicinal settings from the treatment of infectious diseases to oncology [33]. In this review, we will discuss the potential of peptides to modify ER–mitochondrial Ca^{2+} signaling toward therapeutic ends by altering protein interactions and interaction-related functions (see a summary of the peptides discussed below in Fig. 3 and Table 1).

Peptides modulating ER-located Ca^{2+} channels and pumps

ER Ca^{2+} homeostasis and Ca^{2+} release are crucially involved in regulating cell death and cell survival decisions. Studying ER Ca^{2+} homeostasis and Ca^{2+} release has resulted in the development and identification of several peptides targeting the ER Ca^{2+} toolkit. These involve peptides regulating the main Ca^{2+}-release channels: IP_3R and RyR, but also peptides or small proteins that modulate SERCA activity and as such impact the ER filling state.

IP_3Rs

As discussed above, IP_3Rs are regulated by members of the Bcl-2 protein family, well known to control cell death and survival at the mitochondrial level by impacting MOMP [34]. This family consists of pro-and anti-apoptotic Bcl-2 family members that exert functions at several organelles including the ER and mitochondria. At the mitochondria, antiapoptotic Bcl-2 family members (like Bcl-2, Bcl-XL and Mcl-1) target and neutralize the activity of proapoptotic family members (such as the MOMP executioners Bax and Bak, the 'activator' BH3-only protein Bim and the 'sensitizer' BH3-only protein Bad), the major molecular promoters of MOMP, cytochrome C (cyto C) release and apoptosis induction. This interaction occurs via the Bcl-2 homology (BH) domain 3 of the proapoptotic family members and the hydrophobic cleft, formed by the BH1, 2 and 3 domains of the antiapoptotic Bcl-2 family members [28]. The binding interface between these two classes of proteins has long been an object of interest for the development of drugs targeting cancers with upregulated Bcl-2 levels [35]. To this end, many strategies have been pursued: from antagonizing Bcl-2 mRNA to small molecules imitating the BH3 domains of proapoptotic proteins – so-called BH3 mimetics [36]. For instance, ABT-199 (venetoclax) is a Bcl-2 antagonist that mimics the function of the BH3 domain of Bad, a sensitizer BH3-only protein that inhibits Bcl-2 without directly activating Bax/Bak. Venetoclax was recently approved for the clinical treatment of 17p-deleted relapsed chronic lymphocytic leukemia (CLL) patients [37,38].

Antiapoptotic Bcl-2 proteins also play important parts in modulating intracellular Ca^{2+} signaling [39]. These proteins target several Ca^{2+}-transport systems present at different organelles, including the ER [39,40]. For instance, Bcl-2 binds via its BH4 domain to the central regulatory region of the IP_3R, thereby inhibiting IP_3R-mediated Ca^{2+} release [41]. Introducing the BH4 domain of Bcl-2 (BH4-Bcl-2) as a peptide into cells is sufficient to

TABLE 1

Overview of the peptides acting on ER–mitochondrial Ca^{2+} fluxes discussed in this review

Peptide name	Targeted organelle	Molecular target	Effect on ER or mitochondria Ca^{2+} gateways	Potential therapy	Refs
BH4-Bcl-XL	ER and mitochondria	RyRs and VDAC1	Inhibitory	Acute pancreatitis, ALS ischemia–reperfusion, Alzheimer disease, Parkinson disease	[66,103–105, 118]
BH4-Bcl-2	ER	IP_3Rs and RyRs	Inhibitory	Acute pancreatitis, neurodegenerative diseases?	[41–43,65,118]
BIRD-2	ER	IP_3Rs	Stimulatory	DLBCL, CLL, multiple myeloma, follicular lymphoma, ovarian cancer	[46–50]
dTAT-NDP	ER	IP_3Rs	Stimulatory	Breast cancer	[56,57]
Peptide A	ER	RyR	Stimulatory	Myopathies and muscle weakness	[60,61]
Calcins	ER	RyRs	Stimulatory	Myopathies and muscle weakness	[63]
PLB phospomimetics	ER	PLB (SERCA)	Stimulatory	Cardiomyopathies	[79]
DWORF	ER	SERCA	Stimulatory	Cardiomyopathies	[81,82]
N-Ter and LP4 peptide variants	Mitochondria	VDAC1	Inhibitory or stimulatory?	Glioblastoma, lung cancer, breast cancer, HCC	[95,98,99]
NHK1/pHK-PAS	Mitochondria	VDAC1	Inhibitory or stimulatory?	ALS, cervical cancer, breast cancer, colon carcinoma	[119–123]
TAT-FHIT 7-13	Mitochondria	MCU	Stimulatory?	Lung cancer	[126]
BNP	Mitochondria	PI3K (MCU)	Inhibitory	Heart-related diseases	[127]
AIP/AC3-I/CaMKIIN	Mitochondria	CaMKII (MCU)	Inhibitory	Heart-related diseases	[140–143]
mtCsA/NIM-811/Debio-025	Mitochondria	CypD (mPTP)	Inhibitory	Cardiotoxicity	[152–155]
R8:MT	Mitochondria	mPTP	Stimulatory	Multiple solid tumors	[136,160]
RRNY	Mitochondria	Cx43	Inhibitory	Cardiotoxicity	[138,164]

Abbreviations: AC3-1, Autocamtide - 3 Derived Inhibitory Peptide; AIP, Autocamtide-2-Related Inhibitory peptide; ALS, amyotrophic lateral sclerosis; BH4, Bcl-2 homology domain 4; BIRD-2, Bcl-2/IP_3R disruptor 2; BNP, brain natriuretic peptide; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaMKIIN, Ca2+/ calmodulin- dependent protein kinase II inhibitor 2; CLL, chronic lymphocytic leukemia; Cx43, connexin 43; CypD, cyclophilin D; dTAT-NDP, dTAT Nrh BH4 domain peptide; DWORF, dwarf open reading frame; DLBCL, diffuse large B-cell lymphoma; ER, endoplasmic reticulum; HCC, Hepatocellular carcinoma; IP3R, inositol 1,4,5-trisphosphate receptor; LP4, loop 4; MCU, mitochondrial Ca^{2+} uniporter; mPTP, mitochondrial permeability transition pore; mtCsA, mitochondrial-targeted cyclosporin A; NHK1, N-terminal HKI peptide 1; NIM811, N-methyl-4-isoleucine cyclosporine; N-Ter, VDAC1 N-terminal; pHK-PAS, HKII-N-terminal domain penetration accelerating sequence peptide; PI3K, phosphoinositide 3-kinase; PLB, phospholamban; RRNY, octapeptide RRNYRRNY; RyRs, ryanodine receptors; R8:MT, 8 arginines mitochondrial targeting domain peptide; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; TAT-FHIT 7-13, TAT-fragile histidine triad aa 7-13; VDAC1, voltage-dependent anion channel 1.

inhibit IP_3R-mediated Ca^{2+} release and protect cells from proapoptotic Ca^{2+} transfer to the mitochondria [42,43]. In cancers where Bcl-2-protein levels are upregulated, this inhibition of IP_3R-mediated Ca^{2+} release can thus at least in part contribute to their resistance to cell death. In contrast to the BH4 domain of Bcl-2, the BH4 domain of the antiapoptotic Bcl-XL protein did not inhibit IP_3Rs [42]. The role of BH4-Bcl-2 on the IP_3Rs and its resulting protective activity could be exploited for the treatment of neurodegenerative diseases, which have been recently characterized by altered ER–mitochondria Ca^{2+} signaling [44,45].

An IP_3R-derived peptide, Bcl-2/IP_3R disruptor 2 (BIRD-2), was modeled after the BH4-Bcl-2 binding site in the central modulatory region of the IP_3R, which has been pinpointed to a region of 20 amino acids [46,47]. This peptide targets the BH4 domain of Bcl-2 and kills Bcl-2-dependent cancer cells, including diffuse large B cell lymphoma (DLBCL), CLL, multiple myeloma and follicular lymphoma [47–50]. The sensitivity of DLBCL cancer cells toward BIRD-2 correlated with the expression levels of IP_3R2, the isoform with the highest sensitivity to IP_3, and depended on constitutive B cell receptor activity, resulting in increased basal IP_3 production [48,49]. The mechanisms by which BIRD-2 provokes cell death are not yet fully elucidated; although properly filled ER Ca^{2+} stores and Ca^{2+} from the extracellular environment are clearly required [48]. Disrupting the Bcl-2–IP_3R interaction can also be applied as a sensitizing strategy toward BH3-mimetic drugs (e.g., ABT-263 and ABT-199 or venetoclax), as shown in multiple myeloma, follicular lymphoma, small-cell lung cancer cells and DLBCL [50–52]. The underlying mechanism can relate to the ability of BIRD-2 to provoke Ca^{2+} signals that cause upregulation of the activator BH3-only protein Bim [50,52]. A recent publication shows that it can also be used as a sensitizer for classic chemotherapy. Xie *et al.* observed that Bcl-2 overexpression in an ovarian cancer model confers cisplatin resistance [53]. This cisplatin resistance could be overcome by BIRD-2, increasing cisplatin-induced ER–mitochondrial Ca^{2+} signaling. An interesting point is that BIRD-2 itself did not provoke cell death in the ovarian cancer cells [53]. Further discussion on the discovery, mechanism of action and application of BIRD-2 and BIRD-2-mimicking compounds is provided elsewhere [54,55]. Finally, a pro-survival protein homolog of Bcl-2, namely Nrh/Bcl2L10 or Bcl-B, could also bind, via its N-terminal BH4 domain, to a different IP_3R region [56]. In this regard, Nrh has been shown to negatively regulate chemotherapy-induced apoptosis in cancer. Surprisingly here, not an IP_3R-derived peptide but a decoy peptide comprising the sequence of the Nrh BH4 domain (dTAT-NDP) would compete with the full-length Nrh from IP_3Rs and restore apoptosis responsiveness in breast cancer cells [56,57].

RyRs

In skeletal muscle fibers and cardiomyocytes, RyR activation is crucially involved in EC coupling. In both cell types, DHPRs activate the RyR either via a complex network of molecular interactions (skeletal muscles) or via Ca^{2+} influx triggering Ca^{2+}-induced Ca^{2+} release (heart). In skeletal muscle, the molecular determinants underlying RyR1 activation by DHPR have been extensively studied. It is known that the cytosolic II–III loop of the DHPR is crucial for EC coupling. However, whether this loop directly interacts with RyR1 is still unknown [58]. Further research indicated that a short fragment of this II–III loop termed peptide A can induce RyR1-mediated Ca^{2+} release [59]. Efforts have been made to enable potential therapeutic applications of peptide A by increasing its cell permeability [60]. Lipoamino acid conjugation of peptide A increased the cell permeability while maintaining structural and functional properties of the peptide. In addition, recent peptidomimetic approaches have led to the production of several compounds mimicking peptide A activity [61]. As such, these compounds could in the future hold therapeutic value in muscle diseases caused by impaired RyR1 activity. Interestingly, several peptide toxins, termed calcins, present in venom of, for instance, scorpions show similar actions as peptide A on RyR1 gating [62]. In general, calcins are short cell-permeable peptides, which specifically and with high affinity bind to and stimulate RyR1 activity [63]. An example of one of these calcins is imperatoxin A, which shows structural similarity with parts of the II–III loop of the DHPR [64].

Besides IP_3Rs, antiapoptotic Bcl-2 proteins were shown to target RyRs via their BH4 domains [65]. The BH4 domain of Bcl-XL and Bcl-2 (BH4-Bcl-XL; BH4-Bcl-2) are capable of binding to and inhibiting RyR-mediated Ca^{2+} signaling [65,66]. Thus, these peptides hold the potential to counteract hyperactivity of IP_3Rs and/or RyRs underlying pathological conditions. One of these conditions is acute pancreatitis, a disease caused by self-digestion of the pancreas as a result of premature zymogen activation in response to loss of ATP and excessive intracellular Ca^{2+} release [67]. Acute pancreatitis does not only occur in response to high fat and/or alcohol consumption but also as an adverse event in L-asparaginase treatments of children with acute lymphoblastic leukemia [68,69]. Recently, we showed that the BH4 domain of Bcl-2 and Bcl-XL can be used to suppress pathological bile-acid-induced RyR-mediated Ca^{2+} release in pancreatic acinar cells, thus preventing necrosis, a hallmark in the development of acute pancreatitis [70]. This nicely illustrates the therapeutic potential that drugs mimicking BH4 domain functions at ER Ca^{2+}-release channels may hold. In particular, such compounds could be considered in combination with strategies that aim to restore ATP homeostasis in acute pancreatitis, such as galactose treatment [71,72].

In our initial study, in which the binding of Bcl-2 to the RyR was characterized, it was suggested that a stretch of 22 amino acids in the central domain of the RyR was the target for the BH4 domain [65]. Remarkably, this site also contained one of the previously proposed binding sites for FKBP12, another important regulator of RyR activity [73]. Additionally, peptides derived from regions surrounding the recently identified Bcl-2 binding site (aa 2442–2477 RyR1 and aa 2460–2495 RyR2) (56,57) have been exploited with the aim of molecularly characterizing a number of RyR1 and RyR2 mutations underlying malignant hyperthermia (MH) or catecholaminergic polymorphic tachycardia (CPVT), respectively [74]. These peptides were shown to interfere with interdomain interactions of the RyR, thereby increasing RyR-mediated Ca^{2+}, a key feature of MH and CPVT. The authors proposed a model in which MH and CPVT-associated mutations disrupt these interdomain interactions thereby resulting in excessive RyR activity. Drugs that could stabilize the disrupting effects of these mutations on RyR interdomain interactions were proposed to have beneficial effects for treating CPVT and MH.

SERCA

SERCA activity is essential to maintain ER Ca^{2+}-store content enabling efficient Ca^{2+} signaling. In addition, SERCA activity contributes to lowering cytosolic Ca^{2+} levels to basal conditions after Ca^{2+} release has occurred. Different SERCA isoforms exist with SERCA1 being the major isoform in skeletal muscle, SERCA2a in the heart and SERCA2b as the housekeeping isoform present in virtually every cell of the human body [75]. In the heart, SERCA2a transports cytosolic Ca^{2+} back into the SR, promoting relaxation while, by refilling the SR, it prepares the heart for the next contraction. Therefore, it comes as no surprise that SERCA activity is closely regulated in the heart. Several small proteins and peptides that regulate SERCA activity have been identified. These include, phospholamban, sarcolipin, myoregulin and DWORF, which play key parts in regulating SERCA activity in particular in muscle and heart cells [76,77]. Phospholamban, a small protein (52 amino acids) expressed in the heart, for instance binds to and inhibits SERCA2a function [78]. Beta-adrenergic stimulation of the heart results in PKA-mediated phosphorylation of phospholamban, removing it from SERCA2a, promoting its Ca^{2+}-uptake activity. This enhances the rate of refilling of the SR and relaxation of the heart. Conversely, protein phosphatase 1 (PP1)-mediated dephosphorylation of phospholamban results in increased binding and thus inhibition of SERCA. Peptides mimicking these phosphorylation sites (PLB phosphomimetics) have recently been used as decoys for PP1, thereby increasing phospholamban phosphorylation, alleviating SERCA inhibition [79]. Importantly, mutations in phospholamban, which either enhance its inhibitory functions on SERCA or prevent its dissociation from SERCA via PKA-mediated phosphorylation, have been identified and can lead to dilated cardiomyopathy [80].

Recently, an open reading frame, identified on a previously assumed long noncoding RNA, encoding a short peptide named DWORF (34 amino acids) was discovered to modulate SERCA Ca^{2+}-uptake activity [81]. DWORF is endogenously present in the heart and different skeletal muscle groups, where it localizes at the SR and interacts with SERCA. All SERCA isoforms, including skeletal muscle type SERCA1, heart muscle type SERCA2a and the housekeeping isoform SERCA2b, can form a complex with DWORF. By competing with phospholamban and thus antagonizing the effects of phospholamban on SERCA, DWORF can enhance SERCA activity. In this manner, DWORF enhances SR Ca^{2+} uptake and cardiomyocyte contractility. Importantly, DWORF has been used in a mouse model of dilated cardiomyopathy where it was shown to restore cardiac function by increasing SERCA function, thus normalizing Ca^{2+} handling in the SR [82].

Peptides modulating mitochondrial Ca^{2+} gateways

Numerous mitochondria-acting peptides have been developed in the past 15 years, most of which are able to efficiently modulate mitochondrial membrane permeability and, in turn, cell death [83–87]. However, to date, there is scarce to no evidence suggesting that any of these peptide tools would affect either mitochondria or ER Ca^{2+} gateways [36,88–90]. Major exceptions to this apparent rule emerge for peptides, proven to modulate mitochondrial Ca^{2+} uptake by directly or proximally acting on VDAC and MCU.

VDAC1

VDAC1 serves as the major gatekeeper for the passage of metabolites (e.g., Krebs cycle intermediates and glutamate), nucleotides (e.g., ATP/ADP and NAD^+/NADH) and ions such as Ca^{2+} [91]. VDAC1 has a well-deserved reputation as a cell fate regulator because of: (i) its interaction with the Bcl-2 family of proteins and bioenergetics enzymes like hexokinases (HKs) [92]; (ii) its homo- and hetero-oligomerization activity to form cyto-C-permeable channels and therefore promote MOMP [93]; (iii) its ability to build up a fast-track pathway for mitochondrial Ca^{2+} loading by directly facing ER-resident IP_3Rs at the MAMs [12,40]. VDAC1 is composed of 19 antiparallel β-strands organized in a transmembrane β-barrel pore. The N-terminal domain of this unconventional channel is a short α-helical stretch that can flip in and out of the pore lumen allowing VDAC1 oligomerization and PPIs to occur. Additionally, the intracellular interactions of VDAC1 are also modulated by some of the channel's cytoplasm-facing loops, namely LP1, LP3 and LP4 [94].

Many cancers are characterized by alterations in VDAC1 activity, expression and/or functionality, making VDAC1 an interesting drug target. As such, a series of VDAC1-derived peptides has been recently developed for targeting the binding of the channel with pro-survival proteins like Bcl-2, Bcl-XL, Mcl-1 and HKI/II [91]. These peptides, optimized for cell penetration efficiency (e.g., engineered by adding the Antennapedia homeodomain) and in cellulo stability [95] have been shown to promote tumor apoptosis by, at least in part, affecting the pro-survival Ca^{2+}-transport activity of VDAC1. In more detail, short peptides derived from the N terminus of VDAC1 and LP4 (Antp-N-ter and Antp-L14-15, respectively) clearly hindered mitochondrial Ca^{2+} uptake and counteracted lung cancer cell migration [96], probably by blocking the interaction between VDAC1 with Bcl-XL, Mcl-1 [96,97] or HKI [98]. Further research demonstrated that relatively longer versions of these VDAC1-peptide tools, specifically Antp-LP4 and N-Ter-Antp, induce tumor apoptosis at low micromolar concentrations in a diverse set of cancer cell lines [99]. The same group created more-stable, soluble and selective versions of these peptides by introducing D-amino acids, retro-D-amino acids and employing the penetrating sequence of the transferrin receptor (hTfR) [100,101], respectively. The R-Tf-D-LP4 peptide clearly led to increased intracellular Ca^{2+} levels, an event associated with VDAC1 oligomerization, cyto C release and apoptotic cell death [92,99]. Importantly, R-Tf-D-LP4 reduced tumor burden in xenograft mouse models (glioblastoma, lung and breast cancer) and in diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) [99,102]. In addition, peptides derived from the sequences of proteins that are accessories for VDAC1 function have shown potential therapeutic applications. In this regard, Bcl-2 and Bcl-XL are optimal candidates because they have been repeatedly reported to directly bind to and control VDAC activity [103]. Noticeably, the aforementioned BH4 peptides of Bcl-2 and Bcl-XL are capable of differentially modulating VDAC1 activity [104,105]. BH4-Bcl-XL is almost exclusively hampering VDAC1-mediated Ca^{2+} uptake into the mitochondria and therefore renders cells more resistant to proapoptotic Ca^{2+} release from the MAM-located ER [105]. In this molecular scenario, BH4-Bcl-XL action on VDAC1, together with its role at the ER as RyR inhibitor, is exploitable to treat diseases characterized by toxic mitochondrial Ca^{2+} signaling such as

ischemia–reperfusion injuries [106], Alzheimer's disease [107], Parkinson's disease [108] and amyotrophic lateral sclerosis (ALS) [109,110]. Indeed, a great amount of experimental evidence has already been collected showing the protective antiapoptotic effect of BH4-Bcl-XL peptides in several of these diseases [111–118].

Another group of peptide tools that can be ascribed to this class includes peptides developed to bind VDAC1 by mimicking specific protein domains of HKI and HKII. A peptide based on the first 11 N-terminal residues of HKI (NHK1) showed the ability to hamper toxicity in ALS models [119]. NHK1 would impair the interaction between the mutant form of superoxide dismutase 1 (SOD1), SOD1 G93A and VDAC1, in turn adjusting VDAC1-conductance and boosting mitochondrial bioenergetics [119,120]. Conversely, a peptide encompassing the HKII N-terminal domain (pHK) and flanked by a penetration accelerating sequence (pHK-PAS) could trigger apoptosis in multiple cancer models [121–123] by dislodging HKII from mitochondria and inducing mitochondrial dysfunction. However, the precise mechanism of action of these peptides is still controversial and the direct contribution of mitochondrial Ca^{2+} fluxes has not yet been ruled out.

MCU

To cross the outer mitochondrial membrane (OMM), MCU-targeting peptides require additional physicochemical properties, such as an enrichment of basic amino acids and amphipathicity [124,125]. The presence of a positively charged cell-penetrating sequence (e.g., TAT) could further increase the cellular and mitochondrial uptake of these peptides. Their mitochondrial accumulation is driven by the negative potential across the inner mitochondrial membrane (IMM) as well as by their affinity for IMM-specific phospholipids. Some of those peptide tools are presently engineered to further enhance partitioning into mitochondria. Among such peptides, able to affect the Ca^{2+} uptake activity of MCU, TAT-FHIT 7-13, BNP and mtCaMKIINs are particularly noteworthy.

The fragile histidine triad (FHIT) gene encodes for a tumor suppressor that localizes in mitochondria and sensitizes MCU, therefore enhancing Ca^{2+} uptake into the organelle and heightening the effect of apoptotic agents [7]. Later work showed that a HIV-TAT-fused version of FHIT recapitulates the function of the transiently overexpressed protein and induces apoptosis in hepatocellular carcinoma cells [117]. This effect was partly attenuated by the BH4-Bcl-XL peptide, which is prone to hamper mitochondrial Ca^{2+} uptake [105,117]. A short N-terminal-FHIT-derived peptide, TAT-FHIT 7-13, effectively triggered apoptosis in lung cancer cells and sensitized them to chemotherapy [126], hinting toward an effect partially attributable to aberrant mitochondrial Ca^{2+} uptake.

B-type natriuretic peptide (BNP) is a 32-amino-acid neurohormone responsible for circulatory homeostasis which is released from cardiomyocytes in response to ventricular stretch or stress. A seminal study [127] showed that treatment with BNP protected cardiomyocytes from apoptosis and ischemia-reperfusion injury. This protective effect was attributed to the activation of the PI3K pathway and subsequent inhibition of the MCU. Therefore, the authors suggested that BNP-mediated activation of PI3K would be cardioprotective by partially affecting MCU and mPTP opening [127]. Many clinical trials followed and investigated the use of the recombinant form of BNP, for the treatment of various heart-related diseases [128–130]. The peptide has been FDA-approved (nesiritide, Natrecor®) for the treatment of acutely decompensated congestive heart failure, although controversies remain concerning its clinical efficacy.

Modulation of Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) has also been proposed as a therapeutic target to curb MCU activity. CaMKII is a multifunctional Ca^{2+}-stimulated protein kinase, which coordinates the actions of multiple Ca^{2+}-linked signals and cell death pathways in heart pathophysiology [131–133]. A subset of mitochondrially localized CaMKII can phosphorylate MCU at two sites (Ser57 and Ser92), resulting in increased Ca^{2+} uptake [134,135]. Accordingly, a body of evidence shows that CaMKII inhibition attenuates cell death in the heart, probably by reducing MCU-driven Ca^{2+} entry into mitochondria and suppressing mPTP opening [134,136,137]. Furthermore, CaMKII activation appears to be a crucial mediator in the crosstalk between stressed ER and mitochondria via regulating intracellular Ca^{2+} mobilization from ER to cytoplasm [138]. A series of increasingly specific CaMKII inhibitory peptides [139] have been developed, starting from the discovery of the autoinhibitory regulatory segment of CaMKIIα [autocamtide-2-related inhibitory peptide (AIP), autocamtide-3 inhibitor (AC3-I)] [140,141] or later from a screening to detect small endogenous CaMKII-inhibitory proteins (CaMKIIN) [142]. However, the clinical applicability of CaMKII inhibition for heart failure therapy has not yet been tested owing to persistently observed off-target effects and issues with oral bioavailability [143]. All the more, the role of the CaMKII–MCU axis in heart-related diseases like ischemia–reperfusion injury is still under scrutiny [144].

Peptides indirectly affecting ER–mitochondria Ca^{2+} fluxes or homeostasis

This group of peptides has been reported to modulate the mitochondrial Ca^{2+} filling status without affecting the Ca^{2+} gateways of the main organelles. The main player here appears to be mPTP. mPTP was initially thought to be a Ca^{2+}-release channel induced by mitochondrial Ca^{2+} overload. Later, it became clear that mPTP promotes toxic mitochondrial permeabilization, whereas, under certain conditions, it could transiently act as a sort of Ca^{2+} safety valve protecting against matrix Ca^{2+} overload [145–147]. Notably, nanomolar doses of the fungus-derived cyclic peptide cyclosporin A (CsA) are sufficient to desensitize mPTP to Ca^{2+} and to suppress pore opening. This phenotype indicated a role of the mitochondrial matrix protein cyclophilin D (CypD), the main target of CsA, as a constituent of mPTP. However, the precise molecular identity of mPTP remains elusive [145,148]. At the onset of myocardial reperfusion injury, CypD was suggested to promote mPTP opening by interacting with VDAC1-Grp75-IP_3R1 complexes at the MAMs and facilitating the Ca^{2+} transfer from the ER to mitochondria [149–151]. Recently, more CypD-selective and nonimmunosuppressive derivatives of CsA (e.g., mtCsA, NIM-811, Debio-025) were developed as promising cardioprotective agents owing to their observed ability to reduce the deleterious effect of acute myocardial infarction in different models [152]. Although CsA itself showed controversial results in clinical trials for cardioprotection [153–155], these improved versions of CsA have yet to be clinically tested for this therapeutic application. Interestingly, NIM-811

(SDZ 811) and Debio-025 (alisporivir) also appear to significantly reduce hepatitis C virus (HCV) replication and prevent HCV-induced mitochondrial Ca^{2+} overload; the latter being a molecular event recently identified as seminal in HCV-infected hepatocytes [151,156]. Although both CsA-derived peptides had already been clinically tested as relatively safe HCV treatments [157,158] (registration number NCT01446250, NCT00983060), they have so far been approved solely for investigational purposes. An additional set of peptides that encompasses the mitochondrial targeting domain (MTD) of the proapoptotic protein Noxa has instead been proposed to promote mPTP opening and, in turn, mitochondrial Ca^{2+} leak [159]. Specifically, the MTD sequence, flanked by a series of eight arginines at the N terminus (R8:MT), is sufficient to induce an mPTP-dependent intracellular Ca^{2+} spike and necrotic cell death in solid-cancer cell lines resistant to apoptosis as well as in a mouse colon carcinoma xenograft [136,160].

More recently, the octapeptide RRNYRRNY (RRNY) has been put forward as a potential cardioprotective agent by inhibiting mitochondrial Cx43 hemichannels [138]. Connexins, such as Cx43, form, besides gap junctions and plasmalemmal hemichannels (HCs), mitochondrial Ca^{2+}-permeable HCs that can favor mitochondrial Ca^{2+} overload, loss of energetics and ion gradients, in turn leading to cell death [161–163]. RRNY has been designed using the pharmacophore model of the cytoplasmic loop that is responsible for binding the C-terminal tail of Cx43. Thus, RRNY peptide would compete with the cytoplasmic loop for binding to the C-terminal tail, preventing loop–tail interaction and thus the opening of these Cx43 hemichannels (see [164–166] for molecular details about Cx43-specific opening mechanism). Accordingly, RRNY can counteract the detrimental role of mitochondrial Cx43-HCs via a channel-inhibitory activity that translates into a mitigation of mitochondrial Ca^{2+} overload and of infarct size during cardiac ischemia–reperfusion [138]. Very recently, a review has been dedicated toward the therapeutic targeting of connexin-based channels, including the use of connexin-derived peptides [164].

Concluding remarks and future perspectives

In recent times, drug discovery has seen a revival of interest in peptide pharmaceuticals owing to their potential ability to mimic natural pathways more closely and safely when compared with classical small-molecule therapeutics. To date, >100 peptide drugs gained access to the global market for various clinical indications (http://crdd.osdd.net/raghava/thpdb/) [167], with >200 peptides currently in active preclinical or clinical development [168]. Importantly, the latest peptide scaffolds have been generated not only against extracellular targets but also to modulate previously undruggable targets such as intracellular PPIs and signaling hubs [167,169]. Ongoing research efforts in this direction include the development of peptides modulating the pro-cell survival or pro-cell death Ca^{2+} fluxes between ER and mitochondria.

However, it is clear for most of the peptides illustrated in this review that some additional conceptual and technical hurdles are in the way of an effective rational drug design. In particular, the pleiotropy of many of the targets, the complexity of the MAMs interactome and its effects on cell function could give rise to various unexpected clinical effects. More generally, PPIs, including the ones regulating ER–mitochondria Ca^{2+}-fluxes, are inherently transient in nature and partner proteins exhibit weak affinity for each other [170]. This implies that PPI modulators, by only partially mimicking native interaction surfaces, are often characterized by low potency. However, modern advances in medicinal chemistry can be employed to optimize for affinity or specificity and additionally circumvent potential off-target effects caused by the molecular promiscuity of intracellular interactions. In this regard, a lesson can be learnt from the successful story of the peptide-like compound ABT-199 [38]. This drug mimics the function of the BH3 domain of Bad and was optimized post hoc to selectively bind to and inhibit its Bcl-2 target with high affinity and potency. Such multidisciplinary and medicinal-chemistry-guided approaches could lead to the development of highly selective peptidomimetic drugs of tomorrow for many of the still unmet medical needs of today.

Ultimately, the field of ER–mitochondrial contact sites has been expanding fast, meaning that PPIs at the MAMs are unveiled swiftly, presenting new opportunities for further targeted therapy. Apart from the targets discussed above, there are many more intracellular hotspots involved in ER–mitochondria Ca^{2+} crosstalk that would be worthy of further investigation [for a detailed overview see the 2018 cell death and disease collection Mitochondria-Associated Membranes (MAMs) and Pathologies] [171]. For example, peptides could be developed to disrupt the binding between FBXL2 and the IP_3R, to modify the stability and function of IP_3R in cancer cells. Altogether, the promising in vitro and preclinical data collected by using decoy or modulatory peptides acting on the major ER–mitochondria Ca^{2+} gateways call for the prompt development of these tools into actual therapeutics.

Conflicts of interest

The authors declare no competing interests.

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