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Recent advances in the development of Mcl-1 inhibitors for cancer therapy

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ABSTRACT

Dysregulation of the mitochondrial apoptotic pathway controlled by members of the Bcl-2 protein family plays a central role in cancer development and resistance to conventional cytotoxic as well as targeted therapies. Hence, selective inhibition of pro-survival Bcl-2 family of proteins to activate apoptosis in malignant cells represents an exciting anti-cancer strategy. The remarkable clinical performance of the selective Bcl-2 antagonist venetoclax has highlighted the potential for selective inhibitors of the other pro-survival members of the Bcl-2 family, particularly Mcl-1. Here we review the latest progress on the discovery and development of selective inhibitors of Mcl-1 that are undergoing clinical evaluation for cancer therapy.

Contents

1. Introduction

The Bcl-2 family of proteins regulates the mitochondrial apoptotic cell death critical for normal development and tissue homeostasis. Research over the past two decades has demonstrated that evasion of apoptosis is one of the hallmarks of cancer and a major cause of failure to anticancer therapies (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

Members of the Bcl-2 family bear from one to four BH motifs and are grouped in three subsets: (i) pro-survival proteins that contain BH1-4 motifs (Mcl-1, Bcl-2, Bcl-xL, Bfl-1/A1, Bcl-w, Bcl-b); (ii) pro-apoptotic proteins Bak and Bax that also have four BH motifs and are named 'apoptosis effectors' and (iii) pro-apoptotic BH3-only motif proteins also known as 'apoptosis initiators' (Bim, Bid, Bik, Noxa, Puma, Bad, Hrk, Bmf). Interactions between these subsets determine whether cells live or die (Czabotar, Lessene, Strasser, & Adams, 2014). The signaling pathway is initiated by transcriptional and post-translational activation of pro-apoptotic proteins with homology in the BH3 motif-only in response to stress signals such as DNA damage and oncogene inhibition. BH3-only molecules either bind to and activate pro-apoptotic multi-BH motif proteins Bax and Bak or associate with and inactivate pro-survival multi-BH molecules (Cheng et al., 2001; Kim et al., 2006). Activated Bax and Bak homo-oligomerize and form pores in the mitochondrial outer membrane causing cytochrome *c* and other apoptogenic proteins to leak into the cytosol promoting the formation of the apoptosome, activation of caspases, cleavage of cellular proteins and cell death (Fig. 1) (Chen et al., 2015). Conversely, pro-survival BH molecules sequester Bax and Bak monomers preventing homo-oligomerization preserving in this manner the integrity of the mitochondrial membrane (Chen et al., 2015; Kim et al., 2006; Willis et al., 2007).

Abbreviations: ALL, Acute Lymphoblastic Leukemia; AML, Acute Myeloid Leukemia; Bcl-2, B-Cell Leukemia 2; BH, Bcl-2 Homology; CLL, Chronic Lymphocytic Leukemia; i.v., Intravenously; Mcl-1, Myeloid Cell Leukemia-1; MM, Multiple Myeloma; NHL, Non-Hodgkin Lymphoma; NMR, Nuclear Magnetic Resonance; NSCLC, Non-Small Cell Lung Cancer; PDX, Patient-Derived Xenograft; p.o., Per Oral; q.d., Quaque Die; SLL, Small Lymphocytic Leukemia; TCL, T-Cell Lymphoma; TR-FRET, Time-Resolved Fluorescence Resonance Energy Transfer.

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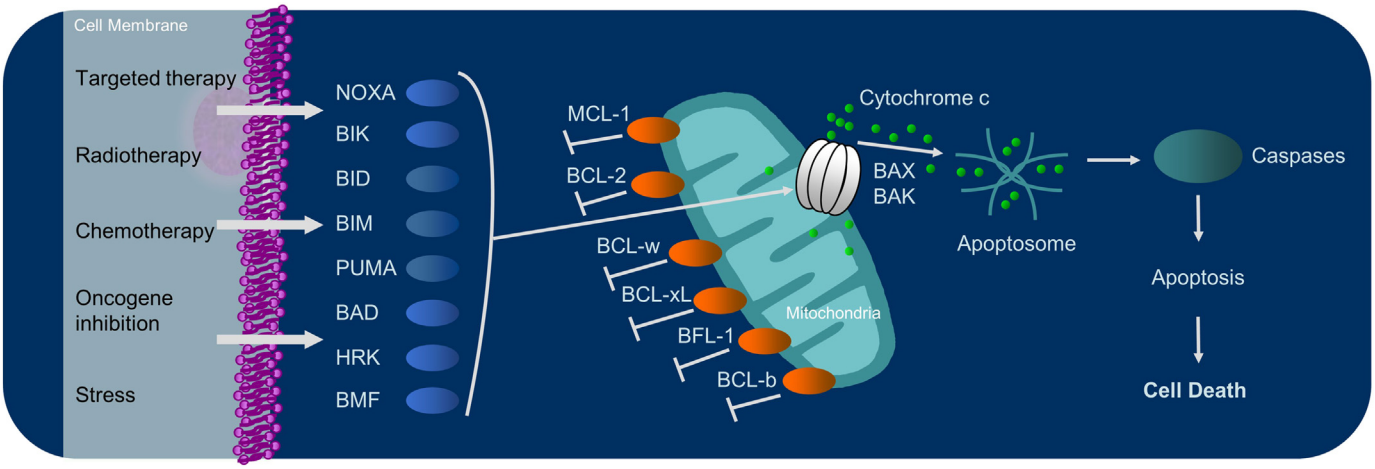


Fig. 1. The mitochondrial apoptotic pathway. Apoptosis is initiated by upregulation of pro-apoptotic BH3-only Bcl-2 proteins (*e.g.* Bim, Bid) in response to various cellular stress signals. The BH3-only proteins then associate with anti-apoptotic Bcl-2 relatives (Mcl-1, Bcl-2, Bcl-xL, Bcl-w, Bfl-1/A1, Bcl-b) unleashing Bak and Bax leading to mitochondrial outer membrane permeabilization, release of cytochrome *c* and other apoptogenic proteins into the cytoplasm, activation of caspases and cell death.

Interaction between Bcl-2 family members occurs by hydrophobic and electrostatic interactions between the BH3 motif of a pro-apoptotic molecule (Bax, Bak, or a BH3-only protein) and the hydrophobic surface groove created by the BH1, BH2 and BH3 motifs of a multi-BH pro-apoptotic or pro-survival protein, termed the BH3 binding site (Czabotar et al., 2014). Hence, the binding affinity and specificity of this network of interactions vary according to sequence in both the BH3 motif and the binding groove (Kong et al., 2018; Kvansakul & Hinds, 2015).

Given that pro-survival Bcl-2 proteins are upregulated in several types of cancers and that many tumors, especially those resistant to cytotoxic therapies, retain a functional apoptotic machinery, it became increasingly evident that inhibition of pro-survival Bcl-2 proteins could be a successful approach to activate apoptosis and trigger cell death in malignant cells (Akagi et al., 2013; Wei et al., 2008; Zack et al., 2013).

Structure-based design led to the discovery of the first highly selective Bcl-2 antagonist venetoclax (Souers et al., 2013). This drug belongs to a class of cancer drugs named 'BH3 mimetics' since they mimic natural BH3 motif inhibitors. The remarkable responses seen in the clinic with venetoclax resulted in its approval by the FDA for the treatment of patients with CLL and validated this drug class for the treatment of cancer (Roberts et al., 2016). Venetoclax has changed the paradigm on how CLL is treated, however, responses to venetoclax monotherapy in other disease indications such as MM and AML are modest, and preclinical studies have shown that other pro-survival Bcl-2 family members including Mcl-1 cause resistance to Bcl-2 inhibition (Bose, Gandhi, & Konopleva, 2017; Konopleva et al., 2016; Kumar et al., 2017; Lin et al., 2016; Tahir et al., 2017; Yecies, Carlson, Deng, & Letai, 2010).

There are several features that make Mcl-1 protein unique compared to its pro-survival Bcl-2 relatives. Mcl-1 expression is regulated at the transcriptional level by trophic factors such as granulocyte-macrophage stimulating factor, epidermal growth factor and cytokines (Chao et al., 1998; Huang, Huang, & Yen, 2000; Jourdan, De Vos, Mechti, & Klein, 2000; Leu, Chang, & Hu, 2000; Wang et al., 1999). At the intracellular level, transcription of Mcl-1 is controlled by several signaling pathways, including the PI3K/AKT, p38/MAPK and STAT3 pathways (Akgul, 2009; Becker et al., 2014; Huelsemann et al., 2014). In addition, >10 miRNA have been identified to modulate *MCL1* translation, including miR-29 and miR-125b, and are reviewed elsewhere (Cui & Placzek, 2018).

Mcl-1 protein levels are tightly controlled by phosphorylation, ubiquitination and protease cleavage resulting in rapid turnover of Mcl-1 modulating its activity in response to pro- and anti-apoptotic stimuli (reviewed in Mojsa, Lassot, & Desagher, 2014). In addition to post-translational regulation, Mcl-1 protein abundance is regulated by its interactions with pro-apoptotic Bcl-2 relatives. These interactions result in displacement of E3-ubiquitin ligases or deubiquitinases leading to Mcl-1 protein stabilization or degradation, respectively (Czabotar et al., 2007; Gomez-Bougie et al., 2011; Nakajima et al., 2016; Warr et al., 2005; Wuillème-Toumi et al., 2007).

The firsts studies linking Mcl-1 activity to cell survival and tumorigenesis were published more than two decades ago (Kaufmann et al., 1998; Lømo, Smeland, Krajewski, Reed, & Blomhoff, 1996). More recent studies have demonstrated that *MCL1* is within one of the most frequently amplified chromosomal regions in human cancers and its expression is often associated with innate and acquired resistance to cytotoxic and targeted therapies (Hu et al., 2013; Li et al., 2015; Shi et al., 2017; Wei et al., 2006; Wei et al., 2008; Wertz et al., 2011; Zack et al., 2013). Increasing molecular evidence indicates that Mcl-1 maintains viability of tumor cells through inhibition of apoptosis in several hematological cancers, including MM and AML, as well as some solid tumors (Glaser et al., 2012; Sieghart et al., 2006; Wuilleme-Toumi et al., 2005; Zhang et al., 2011). This large body of evidence highlights the potential of Mcl-1 inhibitors as anti-cancer therapies.

One important aspect to bear in mind when considering Mcl-1 inhibitors for clinical applications is the key pro-survival role of Mcl-1 in many normal tissues. Mcl-1 is ubiquitously expressed and essential for embryonic development as demonstrated by studies performed in mice with homozygous loss of *MCL1* (Kozopas, Yang, Buchan, Zhou, & Craig, 1993; Rinkenberger, Horning, Klocke, Roth, & Korsmeyer, 2000). Conditional *MCL1* knockout mice showed that Mcl-1 also plays a critical role in the survival of hematopoietic stem cells, lymphocytes and cardiomyocytes, among other normal cells (Opferman et al., 2003; Opferman et al., 2005; Thomas et al., 2013; Wang et al., 2013). However, it is possible that some of the observed effects in normal tissues are caused by chronic depletion of Mcl-1 or other activities besides their anti-apoptotic function and could potentially be mitigated by careful dosing and schedule.

There are several drugs, although not designed to specifically target Mcl-1, that cause downregulation of Mcl-1 activity as part of their mechanism of action. Pan-cyclin-dependent kinase inhibitors, such as flavopiridol, SNS-032, CYC202 (Roscovitine) and dinaciclib, or the selective CDK9 inhibitors BAY1251152 and AZD4573 have been reported to kill cells in part by blocking Mcl-1 transcription (Chen et al., 2009; Cidado et al., 2018; Fu et al., 2011; Gojo, Zhang, & Fenton, 2002; Luecking et al., 2017; MacCallum et al., 2005). Chemotherapeutic drugs such as anthracyclines were found to preferentially repress Mcl-1 transcription to induce apoptosis in tumor cells (Wei et al., 2012). In

addition, the activity of the multi-kinase inhibitor sorafenib in CLL relies on inhibition of Mcl-1 translation for inducing cell death (Huber et al., 2011).

Despite the extensive platform of evidence supporting Mcl-1 as an attractive therapeutic target, the path toward well-characterized small molecule direct inhibitors of Mcl-1 has been slow and muddled with purported direct inhibitors that were subsequently shown to exert the majority of their phenotypic effects in cells *via* alternative mechanisms (Soderquist & Eastman, 2016). Direct inhibitors must disrupt the protein-protein interaction between Mcl-1 and the BH3 motifs of the pro-apoptotic Bcl-2 proteins. The BH3 binding site on Mcl-1 comprises a long shallow and hydrophobic binding groove. Hotspots from the binding site have been identified by selection of mutated Bim peptides, revealing preferences for a conserved aspartate and hydrophobic amino acid side chains at many other positions (Dutta et al., 2010; Stewart, Fire, Keating, & Walensky, 2010). Crystallography of Mcl-1:BH3 peptides has also demonstrated the importance of an interaction between the conserved aspartate and Arg-263 on Mcl-1 (Dutta et al., 2010; Stewart et al., 2010). The nature of the peptide binding site suggested that potent ligands must occupy much of this large lipophilic surface interface and mimic the salt-bridge interaction with Arg-263. As a result, ligands are likely to be large, lipophilic and acidic; features which often result in poor drug-like properties.

In this review, we discuss the approaches that led to the discovery of direct and selective antagonists of Mcl-1, the current status of these small-molecule inhibitors and future directions including their use in combinatorial therapies for treatment of a variety of cancers. This discussion is especially timely now, as the first direct and selective Mcl-1 inhibitors have entered clinical evaluation as monotherapy and in combination with other anticancer drugs.

2. Discovery and development of direct and selective inhibitors of Mcl-1

Over the past two decades, many papers and patent applications have described Mcl-1 inhibitors yet the literature is contaminated with molecules that exert their phenotypic effects through other mechanisms rather than Mcl-1 inhibition (Chen & Fletcher, 2017; Soderquist & Eastman, 2016). Examples of possible alternative mechanisms include Mcl-1 protein degradation, induction of Noxa or cell killing that occurs independently of Bax/Bak activation. However, more recently a series of publications have begun to describe Mcl-1 inhibitors that are suitable tools for understanding structure-activity relationships, further studying target biology *in vitro* and *in vivo*, and some molecules are even under clinical evaluation.

Initial studies on the discovery of direct Mcl-1 inhibitors were performed by researchers at Abbvie utilizing fragment-based lead generation approaches. (Oltersdorf et al., 2005; Petros et al., 2014; Souers et al., 2013; Wendt et al., 2006). The fragment hits were identified from a protein-based NMR screen with ^{13}C-labelled Mcl-1 and, in a theme that will become common, relied on acidic moieties to interact with Arg-263 of Mcl-1. Compounds were optimized for potency to yield 30 nM Mcl-1 binders (Fluorescence Polarization Assay) enabling the determination of the first crystal structures for small-molecule: Mcl-1. However, despite this optimization no cellular activity was reported presumably because these compounds were not potent enough or lacked the properties necessary for cellular penetration.

2.1. Mcl-1 inhibitors derived from 2-carboxylic acid indoles

2.1.1. A-1210477

Researchers at Abbvie also published a series of indole-2-carboxylic acids identified from a single-hit in a high-throughput screen that resulted in the discovery of A-1210477 (Bruncko, Song, Ding, Tao, & Kunzer, 2008; Elmore et al., 2008; Leverson et al., 2015). A-1210477 is a potent binder of Mcl-1 (K_i = 0.45 nM) with good selectivity over the other Bcl-2 family proteins investigated (over 250-fold *vs.* Bcl-2 and Bcl-xL) that causes disruption of the Mcl-1:Bim complex in cells (Table 1). Target engagement was demonstrated by a dose-dependent increase in Mcl-1 protein levels across a panel of cell lines upon treatment with A-1210477. This activity has been shown with more potent and selective Mcl-1 inhibitors and is discussed later in this article. Only cell lines that were killed by siRNA-mediated Mcl-1 depletion showed sensitivity to A-1210477 giving further evidence that cell death was caused by inhibition of Mcl-1. No *in vivo* activity was shown for A-1210477, which is likely due to the low micromolar potency of this compound in cell viability assays, even in the most sensitive cell lines reported.

The indole-2-carboxylic acid scaffold has been further developed by researchers at AstraZeneca (*vide infra*) as discussed later in this review, the Broad Institute and Bayer (Wagner et al., 2017).

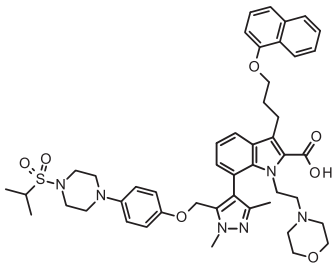
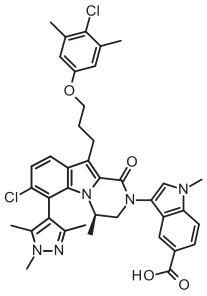
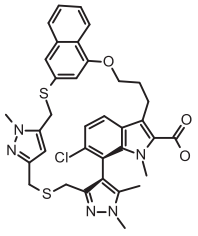
2.1.2. VU661013

Early publications demonstrated the strength of the fragment-linking approach to derive compounds with high biochemical potency and selectivity for Mcl-1 with similarities to the indole-2-carboxylates described by the group at Abbvie (Friberg et al., 2013; Leverson et al., 2015; Shuker, Hajduk, Meadows, & Fesik, 1996). The high affinity of these ligands enabled protein-ligand crystal structures highlighting an induced-fit pocket in Mcl-1 which accommodated a lipophilic substituent. Subsequent publications sought to expand the chemical scope, improve potency further, and reduce plasma protein binding properties that culminated in the discovery of VU661013 (Lee et al., 2017; Pelz et al., 2016; Ramsey et al., 2018; Shaw et al., 2018; Zhao et al., 2017). VU661013 has high binding affinity for Mcl-1 (K_i = 0.097 ± 0.030 nM) with >7000-fold lower affinity for Bcl-2 and Bcl-xL (Table 1). Cell killing activity of VU661013 was demonstrated in a subset of AML cell lines with GI_{50} values at 48 h between 150 and 500 nM for the most sensitive ones. Analysis of Bcl-2 family protein levels *vs.* sensitivity to Mcl-1 inhibition failed to demonstrate an association. Enhanced cell killing was observed for VU661013 combined with venetoclax even in cell lines with innate and/or acquired resistance to VU661013, and in primary AML samples obtained from patients at pre-treatment or after venetoclax + Low Dose Cytarabine failure. *In vitro* activity for VU661013 and venetoclax could be predicted by BH3 profiling, a dynamic *in vitro* bioassay that allows to dissect the anti-apoptotic dependence by measuring cytochrome *c* release as a marker of apoptosis induction (Montero & Letai, 2018).

These *in vitro* findings translated into *in vivo* antitumor efficacy as demonstrated in disseminated xenograft mice models derived from AML cell lines or primary AML cells from patients. In these studies mice were dosed intraperitoneally with VU661013 monotherapy daily for 21 days, or co-dosed with VU661013 intraperitoneally daily and venetoclax p.o. daily at sub-efficacious doses for each of these agents. Studies performed in mice engrafted with human $CD34^+$ umbilical cord blood-derived cells showed that although VU661013 combined with venetoclax decreased human $CD45^+$ cells, a marker of human chimerism, the number of human $CD34^+$ stem and progenitor cells remained unaffected. Lack of activity in non-target tissues should be put in the context of the lower binding affinity for mouse Mcl-1 (mouse Mcl-1 K_i = 7.37 ± 0.06 nM). This drop-off in activity between mouse and human Mcl-1 has also been shown for other selective Mcl-1 inhibitors as described later in this review, and it is caused by differences in the amino acid sequences in the ligand binding pocket in mouse *vs.* human Mcl-1 (Zhao et al., 2018).

No clinical plan has been disclosed for VU661013 although Vanderbilt University and Boehringer Ingelheim have announced an expansion in their partnership with the aim of bringing an Mcl-1 inhibitor to the clinic (https://www.boehringer-ingelheim.com/press-release/expansion-cancer-research-vanderbilt-university).

Table 1
Mcl-1 inhibitors derived from 2-carboxylic acid indoles.

Compound, chemical structure and institution	Potency (TR-FRET)	Clinical trials, patient population, dosing route and schedule	References
 **A-1210477** Abbvie	Mcl-1 K_i = 0.45 nM Bcl-2 K_i = 0.13 μM Bcl-xL K_i > 0.66 μM	*Not selected*	(Bruncko et al., 2015; Leverson et al., 2015)
 **VU661013** Vanderbilt	Mcl-1 K_i = 0.097 nM Bcl-2 K_i = 0.73 μM Bcl-xL K_i > 40 μM	*Unknown*	(Ramsey et al., 2018)
 **AZD5991** AstraZeneca	Mcl-1 K_i = 0.2 nM Bcl-2 K_i = 6.8 μM Bcl-xL K_i = 18 μM Bcl-w K_i = 25 μM Bfl-1 K_i = 12 μM	NCT03218683; MM, CLL, lymphomas, Richter's syndrome; monotherapy; i.v. dosing, schedule not defined.	(Tron et al., 2018)

2.1.3. AZD5991

Following previous publications on *in vitro* probe molecules, a team at AstraZeneca recently disclosed the structure-based optimization of a series of indole-2-carboxylic acids that resulted in the discovery of the potent and selective macrocyclic Mcl-1 inhibitor, AZD5991 (Akcay et al., 2016; Johannes et al., 2017; Tron et al., 2018). AZD5991 is a sub-nanomolar binder of human Mcl-1, although potency was reduced against the rodent isoforms, in line with previous studies (Caenepeel et al., 2018; Kotschy et al., 2016; Zhao et al., 2018).

Inhibition of Mcl-1 by AZD5991 caused rapid disruption of the Mcl-1:Bak complex that resulted in activation of the mitochondrial apoptotic pathway and cell death in sensitive cells. As expected from an inducer of intrinsic apoptosis, this process was abolished by depletion of Bak. In agreement with data published for other selective Mcl-1 inhibitors, treatment with AZD5991 led to an increase in Mcl-1 protein abundance in cell lines with intermediate-low sensitivity to AZD5991. In contrast, Mcl-1 protein levels were decreased by AZD5991 in sensitive cell lines by a mechanism dependent on caspase activity.

AZD5991 showed preferential activity in hematological cell lines, and subsets of NSCLC and breast cancer cell lines, as shown for other selective Mcl-1 inhibitors mentioned later in this article (Caenepeel et al., 2018; Kotschy et al., 2016). Close correlation was seen between the cell growth inhibitory activity and the drug's ability to induce caspase activation in these cells highlighting that the cytotoxic effect of AZD5991 is caused by on-target mechanism. *Ex vivo* assays demonstrated that AZD5991 is also active in inducing apoptosis in primary cells from MM patients (Tron et al., 2018).

In vivo, AZD5991 exhibited rapid activation of mitochondrial apoptosis with dose-response induction of cleaved caspase-3 and cleaved PARP in the tumor. A single i.v. infusion of AZD5991 caused potent anti-tumor activity with complete tumor regression in several AML and MM mouse and rat xenograft models at tolerated doses. AZD5991 also displayed activity in bone marrow as demonstrated in a disseminated xenograft AML model (Tron et al., 2018).

Anti-tumoral activity for AZD5991 in MM models was enhanced by combination with the proteasome inhibitor bortezomib. Consistent with findings reported for other selective Mcl-1 inhibitors, combination with venetoclax enhanced sensitivity to AZD5991 in AML models *in vitro* and *in vivo*, even in models that were resistant to venetoclax and AZD5991 monotherapies (Tron et al., 2018).

In addition, AZD5991 displayed single agent activity in TCL cell lines and PDX models *in vivo* (Koch et al., 2019). Activity of AZD5991 correlated poorly with Bcl-2 family members RNA or protein levels but could be reliably predicted by BH3 profiling (Montero & Letai, 2016). In studies performed with TCL PDX models that were found to be dependent on Mcl-1 based on BH3 profiling, AZD5991 achieved survival improvement as both a single agent and in combination with CHOP chemotherapy.

Although a detailed characterization of the preclinical tolerability profile for AZD5991 has not been published yet, studies performed in the murine *Eμ-Myc* lymphoma model showed that AZD5991 has antitumor efficacy at tolerated doses in a model where AZD5991 is expected to have similar activity in host *vs.* tumor cells (Tron et al., 2018).

Safety and clinical activity of AZD5991 is being evaluated in a phase 1 clinical trial in patients with relapsed or refractory MM, NHL, TCL, CLL, Small Lymphocytic Leukemia and Richter's Syndrome (ClinicalTrials.gov identifier NCT03218683) (Table 1). This clinical study was initiated using i.v. infusion of the drug.

2.2. Non-indole acid Mcl-1 inhibitors

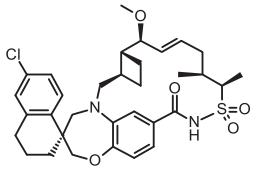
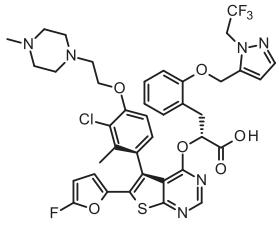
2.2.1. AMG-176 and AMG-397

Using structure-based drug design and conformational restriction as a guiding principle, a team at Amgen optimized a series of spiromacrocyclic molecules that led to the discovery of AMG-176 (Caenepeel et al., 2018). AMG-176 has picomolar affinity for human Mcl-1 and minimal binding affinity toward Bcl-2 and Bcl-xL. Studies performed with the related analog AM-8621 showed that this tool compound disrupts the interaction between Mcl-1 and pro-apoptotic Bcl-2 family members resulting in increased Mcl-1 protein half-life and Bak/Bax-dependent apoptosis and cell death (Table 2). Greater sensitivity to AM-8621 was seen in hematological cell lines, including MM, AML, B-cell lymphoma and subsets of ALL and Burkitt lymphoma. Genomic analysis in a panel of 952 tumor cell lines found no correlation between the potency of AM-8621 and *MCL1* mRNA expression, but an inverse correlation with the levels of *BCL2L1* (that encodes Bcl-xL) mRNA. Conversely, high expression of *BAK* was predictor of sensitivity to AM-8621. These findings are not unexpected given the anti- and pro-apoptotic function of Bcl-xL and Bak, respectively. *In vivo* studies performed with the clinical-stage molecule AMG-176 administered orally showed rapid and dose-dependent induction of intrinsic apoptosis with activation of Bak, induction of cleaved caspase-3 and cleaved PARP by 2 h post-treatment. *In vivo*, AMG-176 dosed p.o. once or twice weekly elicited antitumor activity with disease regression in a subcutaneous xenograft model of MM and in disseminated models of AML and MM (Caenepeel et al., 2018).

Given that AMG-176 has about 1000-fold reduced affinity for mouse *vs.* human Mcl-1, a human *MCL1* knock-in mice where *MCL1* was replaced with its human homolog was utilized to assess tolerability and effects on normal cells at efficacious doses (Caenepeel et al., 2018). AMG-176 caused dose-dependent reduction in B cells in the blood and bone marrow at exposures required for eliciting antitumoral activity. In addition, statistically significant decrease in monocyte and neutrophil counts were observed in blood and bone marrow with no evidence of systemic toxicity based on body weight changes. These data generated in the human *MCL1* knock-in model suggested that a therapeutic window could be achieved for AMG-176, and that reduction in these hematological cell types could be used as pharmacodynamic endpoints (Caenepeel et al., 2018).

Combination of AMG-176 with the proteasome inhibitor carfilzomib showed improved anti-tumoral activity over either single agent alone in an orthotopic model of MM, in agreement with data published for AZD5991 (Tron et al., 2018). In addition, cell killing activity of AM-8621 in AML cell lines and primary samples from AML patients was enhanced by combination with standard cytotoxic AML drugs (Caenepeel et al., 2018). However, greater combination benefit was seen for AM-

Table 2
Mcl-1 inhibitors not derived from indole acid scaffold.

Compound, chemical structure and institution	Potency (method)	Clinical trials, patient population, dosing route and schedule	References
 **AMG-176** Amgen	Mcl-1 K_i = 0.06 nM (TR-FRET) Bcl-2 K_i = 0.95 μM (TR-FRET) Bcl-xL K_i = 0.7 μM (TR-FRET)	NCT02675452; MM, AML; monotherapy and undefined combinations; i.v. dosing, schedule not defined NCT03797261; AML, NHL; combination with venetoclax p.o. and AMG-176 i.v.	(Caenepeel et al., 2018)
Structure not disclosed AMG-397 Amgen	*Not disclosed*	NCT03465540; MM, NHL, AML; p.o. dosing, q.d. for 2 consecutive days, then 5 days off, weekly.	*Not disclosed*
 **S63845** Servier/Vernalis	Mcl-1 K_d = 0.19 nM (SPR) Bcl-2 K_i > 10 μM (FP) Bcl-xL K_i > 10 μM (FP)	*Not selected*	(Kotschy et al., 2016)
Structure not disclosed S64315/MIK665 Servier/Novartis	*Not disclosed*	NCT02979366; AML, Myelodysplastic Syndrome; monotherapy; i.v. infusion over 30 min, once weekly. Starting dose = 50 mg NCT02992483; MM, Lymphoma; monotherapy; i.v. infusion NCT03672695; AML; combination with venetoclax p.o. and i.v. infusion of S64315 2 to 4 h after venetoclax intake once every week.	*Not disclosed*

8621 or AMG-176 when co-dosed with venetoclax, as demonstrated in AML cell lines and primary samples, and in an orthotopic model of AML. Administration of venetoclax and AMG-176 to human *MCL1* knock-in mice at doses and schedule selected based on efficacy observed in an AML model was well tolerated while showing significant reduction in the number of B cells and monocytes.

Cell killing activity for AMG-176 was also seen in few solid tumor cell lines, most of them derived from breast cancer and NSCLC, although its potency was modest (Caenepeel et al., 2018). Recent studies have shown that Bim induction by Mek inhibition using trametinib enhances the activity of AMG-176 or the analog AM-8621 in *KRAS*-mutant NSCLC models *in vitro* and *in vivo* (Nangia et al., 2018). These findings support the notion that inhibition of oncogenic drivers and induction of pro-apoptotic Bcl-2 proteins increase dependency on Bcl-2 pro-survival proteins, Mcl-1 in particular, that can be exploited therapeutically.

Safety and clinical activity of AMG-176 monotherapy is undergoing evaluation in patients with relapsed or refractory MM and AML (ClinicalTrials.gov identifier NCT02675452) and in combination with venetoclax in patients with AML and NHL (ClinicalTrials.gov identifier NCT03797261). Although AMG-176 was administered orally in preclinical studies, i.v. infusion is used in the clinic (Table 2).

A clinical trial is also evaluating AMG-397, the first Mcl-1 inhibitor dosed orally in the clinic. This study was initiated administering AMG-397 orally once daily for two consecutive days followed by five days break at a weekly interval in patients with MM, AML and NHL (ClinicalTrials.gov identifier NCT03465540). The chemical structure of AMG-397 has not been released yet.

2.2.2. S63845 and S64315/MIK665

An NMR-based fragment screen and subsequent structure-guided drug design conducted by a team at Servier and Vernalis yielded S63845, the first potent and selective Mcl-1 inhibitor with *in vivo* activity that was reported in the literature (Kotschy et al., 2016). For comparison, S63845 has about 20-fold higher affinity for Mcl-1 than A-1210477 and no detectable binding to Bcl-2 or Bcl-xL (Table 2). Binding of S63845 to mouse Mcl-1 is weaker than to human Mcl-1 with an approximately 6-fold reduction in K_d as determined by Surface Plasmon Resonance. The co-crystal structure of S63845 bound to Mcl-1 revealed that the carboxylate moiety of this inhibitor establishes a strong interaction with Arg-263, a residue known to anchor binding of inhibitors of other anti-apoptotic Bcl-2 family members (Czabotar et al., 2014). As shown with AM-8621 and AZD5991, S63845 increased Mcl-1 protein abundance in cells by extending its protein half-life with no effect on *MCL1* mRNA levels. Cell assays demonstrated that S63845 induces Bax/Bak-dependent apoptosis and kills hematological cell lines (MM, lymphoma, Chronic Myeloid Leukemia, AML, T-ALL) with IC_{50} values <100 nM (Kotschy et al., 2016; Li, He, & Look, 2018; Manzano et al., 2018). These findings translated into an antitumor response *in vivo*; one cycle of i.v. infusion of S63845 once daily for 5 consecutive days caused complete tumor regression in MM and AML subcutaneous tumor models and in the lymphoma disseminated mouse model *Eμ-Myc* (Kotschy et al., 2016). Consistent with genomic analysis performed with AM-8621, gene expression analysis in a panel of hematological cancer cell lines showed that sensitivity to S63845 does not correlate with *MCL1* mRNA expression, but it is inversely correlated with the levels of *BCL2L1* mRNA. In addition, S63845 showed enhanced induction of apoptosis and cell killing in preclinical *in vitro* and *in vivo* AML models, and primary samples from AML patients when combined with venetoclax (Moujalled et al., 2018). Consistent with the data reported for AMG-176, this activity was superior to combination with standard cytotoxic AML drugs. Combination of S63845 with venetoclax also showed enhanced cell death in T-ALL cell lines and in a zebrafish model of T-ALL (Li et al., 2018).

Although most solid-tumor cell lines tested were resistant to S63845 as a single agent, few NSCLC, breast cancer and melanoma cell lines showed moderate sensitivity with IC_{50} values <1 μM (Kotschy et al., 2016). This cell killing activity of S63845 was enhanced by co-treatment with therapeutic agents known to prime cells for apoptosis by increasing the expression of the pro-apoptotic protein Bim. Studies performed in breast cancer cell lines and PDX models demonstrated that S63845 has increased antitumor activity when combined with chemotherapy in triple-negative breast cancer and with HER2-targeted therapies (lapatinib, trastuzumab) in models of HER2-amplified breast cancer (Merino et al., 2017).

Initial tolerability studies for S63845 were performed in mice and had limited clinical translatability given the lower affinity of this drug for mouse Mcl-1 *vs.* human Mcl-1 (Kotschy et al., 2016). However, a more recent study utilizing a humanized Mcl-1 mouse strain where *MCL1* was replaced with its human homolog, an approach similar to the one used for evaluating tolerability of AMG-176, has provided a more accurate prediction of efficacy and tolerability for S63845 for clinical translation (Brennan et al., 2018). As expected, the maximum tolerated dose of S63845 in this model was lower than in wild-type mice (12.5 *vs.* 40 mg/kg) with reduction in the number of B cells in blood, bone marrow and spleen three days after treatment but normalized at seventeen days post-treatment, as reported for AMG-176. Neutrophil counts were reduced in the bone marrow although this did not reach statistical significance. No changes were observed in the number of $CD4^+$ and $CD8^+$ T cells. S63845 administered at 12.5 mg/kg caused disease regression in the humanized Mcl-1*; Eμ-Myc* lymphoma model with a cure rate of 60% at this dose. Tumor-free survival increased to almost 100% in this model when combined with cyclophosphamide. Together, these data suggest a favorable safety window for S63845 (Brennan et al., 2018).

S63845 was not selected for clinical evaluation and instead a phase 1 clinical trial was launched with the Mcl-1 inhibitor S64315/MIK665. These trials are ongoing in patients with relapsed and/or refractory MM, lymphoma, AML and Myelodysplastic Syndrome (ClinicalTrials.gov identifiers NCT02979366 and NCT02992483) (Table 2). The chemical structure for S64315/MIK665 has not been disclosed yet. These clinical studies were initiated using i.v. infusion of the drug once every week. Supported by preclinical findings obtained with S63845, S64315/MIK665 is also being tested in combination with venetoclax in patients with AML (ClinicalTrials.gov identifier NCT03672695) (Moujalled et al., 2018). The initial treatment regimen in this trial comprises administration of venetoclax orally once a day and i.v. infusion of S64315/MIK665 2 to 4 h after venetoclax intake once every week.

3. Future consideration and conclusions

Over the past few years, considerable progress has been made in the discovery of potent and selective Mcl-1 inhibitors, with four compounds undergoing testing in phase 1 clinical trials. Innovative medicinal chemistry and structure-based optimization coupled with a deep understanding of Mcl-1 biology were essential in enabling this progress that resulted in the discovery of Mcl-1 antagonists with high potency and selectivity capable of causing on-target cell death in several preclinical cancer models.

Many of these inhibitors share several chemical features: (i) An acidic moiety that interacts with Arg-263 of Mcl-1. (ii) Chirality, whether through traditional differentially substituted sp^3 carbon centers (VU661013, AMG-176) or through atropisomerism (S63845, AZD5991). This, together with other structural attributes, results in a scaffold which is rigid and pre-organized for binding to Mcl-1 with rapid on-rate driving high affinity. (iii) Chlorine atoms that improve affinity for Mcl-1; in the case of S63845, VU661013 and AZD5991, there is a specific interaction between the chlorine atoms and the backbone carbonyl of Ala-227, whereas with AMG-176 the chlorine atom is buried in the induced fit hydrophobic pocket.

A common feature among direct and selective Mcl-1 inhibitors is their preferential activity and rapid induction of apoptosis in MM, leukemia and lymphoma-derived preclinical models including those with

genomic features associated with poor clinical outcomes. These findings provided the rationale for testing these molecules in these disease indications in the clinic using discontinuous dosing strategies that provide more flexibility to mitigate potential on-target toxicities without compromising efficacy.

Although anti-tumoral activity was observed with these agents dosed as single agents in hematological cancer models, the use of these drugs in combination with targeted therapies (*e.g.* venetoclax, proteasome inhibitors) or chemotherapies (*e.g.* CHOP, hypomethylating agents) enhanced the depth and durability of the response and was able to elicit activity in models that were resistant to monotherapy (Caenepeel et al., 2018; Koch et al., 2019; Moujalled et al., 2018; Ramsey et al., 2018; Tron et al., 2018). These findings suggested that drug combinations could deepen the response, prolong the durability of the treatment benefit and potentially prevent development of resistance in the clinic. These combination approaches, if successful in the clinic, might also allow patients to come off treatment for extended periods of time.

Given that most solid tumor malignancies are predominantly refractory to Mcl-1 inhibition and Mcl-1 causes resistance to anti-cancer therapeutics in most of these diseases, the value of Mcl-1 inhibitors in these settings clearly resides in their ability to enhance the apoptotic response induced by other anti-cancer drugs known to prime cells for apoptosis. This has been already demonstrated preclinically in several breast cancer and NSCLC models (Merino et al., 2017; Nangia et al., 2018).

Mcl-1 levels are not predictive of response, but it was proposed that sensitivity to Mcl-1 inhibition could be predicted based on the expression levels of *BCL2L1* and *BAK* mRNA (Caenepeel et al., 2018; Kotschy et al., 2016). However, since Mcl-1 activity is tightly regulated at the protein level by modulation of protein turnover and association with pro-apoptotic Bcl-2 proteins, perhaps a more accurate method for determining Mcl-1 dependency and sensitivity of patient samples is the use of dynamic BH3 profiling, or indeed treatment of patient samples with well-validated BH3 mimetic drugs. These approaches have already been validated for Mcl-1 inhibitors in several cell types (Caenepeel et al., 2018; Koch et al., 2019; Moujalled et al., 2018; Ramsey et al., 2018; Tron et al., 2018). Identification of biomarkers that can help identifying sensitive tumor types and likely responders to Mcl-1 inhibitors, as a monotherapy and in combination with other anti-cancer therapeutics, will be essential for the successful development of this new drug class.

Most of the Mcl-1 inhibitors tested in patients are being administered by i.v. infusion. This dosing route allows precise and flexible control over the extent and duration of target coverage and reduces inter-patient variability compared with other administration routes. These factors could prove useful in reducing expected on-target toxicities for these agents. However, oral dosing would allow for more continuous coverage and is more convenient for patients, so it will be interesting to see how each of these compounds and their dosing regimens are optimized for safety and efficacy in the clinic.

Even though no severe toxicities have been reported for Mcl-1 inhibitors at efficacious and pharmacodynamically active doses in the two independent studies performed in humanized Mcl-1 mouse strains, preclinical studies are not always able to predict clinical toxicity (Brennan et al., 2018; Caenepeel et al., 2018). Data from the ongoing phase 1 clinical studies will be critical for understanding the safety margin that Mcl-1 inhibitors can achieve in humans and the clinical management needed for safe administration of these drugs.

After several years of investigation, drug-discovery scientists have succeeded in generating potent and selective inhibitors of Mcl-1 with promising preclinical activity, both as a monotherapy and in combination with other drugs. We envision that translational medicine guided by discoveries made in the laboratory and the clinic will be essential for the successful development of these BH3 mimetics. Having several Mcl-1 antagonists with different drug profiles (route of administration, pharmacokinetics, dosing schedule) undergoing clinical testing will hopefully result in at least one of these therapies being approved, adding another arrow to the quiver and a valuable treatment option for patients.

Conflict of interest statement

Alexander W. Hird and Adriana E. Tron are current or former employees and shareholders of AstraZeneca.

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