

Apoptotic agents

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Activation of apoptosis (programmed cell death) is a highly efficient means of tumour suppression frequently hijacked in lung cancer, and is a major goal of cancer drug therapy. When this can be achieved in the clinic, it is associated with durable disease control. Targeting the core apoptosis pathway has been a research goal since its initial discovery, and outstanding research endeavours have been translated into discovery of a new class of potent, targeted “apoptotic agents”. Despite this, early phase II clinical trials have not met with initial expectations. This review addresses the challenges and significant potential, in the light of recent discoveries, for personalising therapy with apoptotic agents as a basis for improving outcomes in lung cancer.

Apoptosis: a primer

The core apoptosis pathway constitutes a genetically hardwired, and highly regulated mechanism for ensuring cellular demise. It plays a critical role in development, but is hijacked by cancer cells, as an essential transforming process during tumor evolution (1). Apoptotic cell death involves three key events; firstly, an initiation phase engaged by a stimulus such as cellular damage, stress, or inhibition of critical growth factor pathways. Secondly, a commitment phase in which an irreversible decision to initiate apoptosis is made. Mitochondria play a critical role in this phase. Thirdly, the execution phase involving cellular demise. Much is understood regarding the regulation of the apoptotic pathways, in particular, the interplay of the BCL2 proteins which orchestrate the signalling of these first and second phases.

The BCL2 family are composed of pro- and antiapoptotic members which physically interact to govern the initiation of apoptosis (2-4). This event is regulated by

the oligomerisation of multidomain proapoptotic BCL2 proteins BAK and BAX, which constitutively reside in the outer mitochondrial membrane and/or cytoplasm respectively (2,5,6). The trigger for oligomerisation is the BH3-only domain protein sub-family (which comprises at least 8 proteins - BID, BIM, PUMA, BAD, NOXA, BFM, BNIP3, and HRK). BH3 proteins are activated either by transcriptional upregulation e.g., Death receptor triggered cleavage of BID, P53 driven upregulation of NOXA/PUMA) or post-translational modification of BIM by phosphorylation). These proteins then cause apoptosis by either directly triggering oligomerisation of BAX/BAK (BID,BIM,PUMA) or releasing BAX/BAK from members of the prosurvival BCL2 family (BCLX, BCL2, BCLW, MCL1, A1) (7-12). The propensity of BAX/BAK to oligomerise is governed by the ratio of prosurvival to proapoptotic proteins. Cancer cells appear to constitutively activate BH3 proteins (13-15). In order to protect against apoptosis, selection for amplification of prosurvival BCL2 family proteins BCLX and MCL1 occurs as a common event (16). Amplification is associated with dependency which may be therapeutically tractable as discussed further on.

BAX/BAK oligomerisation causes permeabilisation of the outer mitochondrial membrane, releasing multiple pro-apoptotic factors into the cytosol (17-20). This is the event which constitutes irreversible commitment to death - the beginning of the end for the cancer cell. Cellular demolition is executed by the caspases, a family of zymogens which are post-translationally modified leading to their activation (21,22).

Apoptosis and therapeutic outcomes in lung cancer

In recent years, it has become clear, that to achieve

effective outcomes in cancer therapy, induction of apoptosis appears to be a critical requirement. This is borne out in the dramatic radiological regressions associated with inhibition of non-squamous non-small cell lung cancer, harbouring either somatic mutations of the epidermal growth factor receptor (23,24) or an anaplastic lymphoma kinase fusion protein (EML4-ALK) (25-27). These so-called “driver oncogenes” constitutively activate, and lead to dependency on, growth factor signalling pathways involving phosphoinositide 3 kinase/AKT/mTOR and mitogen activated protein kinase (MAPK) axes (28). As a consequence, these pathways constitutively phosphorylate and suppress the BH3 only protein BIM. Following the inhibition of mutated receptor EGFR or ALK receptor tyrosine kinases, BIM is unleashed, leading to activation of BAX/BAK and apoptosis (29-33). Indeed, BIM expression is a prerequisite for clinical activity (34,35). This new paradigm involving targeting of driver oncogene addiction has shown that the mitochondrial apoptosis pathway is fully functional in NSCLC, and that provided a driver oncogene dependency can be identified, mitochondrial apoptosis can be efficiently activated leading to significant improvement in clinical outcome. With the most comprehensive genomic landscape studies to date having recently defined the extent of common somatic mutations in lung cancer (36-38), it is likely that many more clinically tractable oncogene addictions will be validated as effective targets for inducing apoptosis efficiently.

Personalising anti-apoptotic BCL2 inhibition

Prosurvival BCL2 proteins suppress BAX/BAK activation by sequestering both of these multidomain proteins and/or BH3 only proteins (2). The first and most specific inhibitor of BCL2/X/W was ABT-263 (Abbott) (39). Phase II studies were conducted in small cell lung cancer, based on preclinical evidence of addiction to BCL2. However, limited efficacy was observed (40). Why was this? MCL1 is a widely overexpressed prosurvival protein; indeed it is one of the most commonly amplified genes in cancer (16). MCL1 efficiently overcomes the proapoptotic effects of ABT263 and may play a role in clinical drug resistance (41-45). Importantly, the prosurvival BCL2 family addiction observed in cell lines and xenografts was not borne out in heterotransplants nor patients, suggesting that SCLC may not be “predominantly BCL2/BCLX” addicted in the clinical setting. Furthermore, it is clear in SCLC that the tumour microenvironment could significantly impact

cancer cell biology by significantly attenuating apoptotic susceptibility (46,47), something which has been modelled preclinically in NSCLC and mesothelioma (48,49). Nevertheless, patient subgroup analysis showed that in patients with high circulating Pro-GRP, encoded by a gene neighbouring BCL-2 and co-amplified in SCLCs with BCL2 amplification, there was a greater response rate (40). This suggests, that in the context of BCL2 amplified SCLC, AB263 may exhibit single agent activity consistent with a degree of sensitivity. This genetic event exists only in a proportion of patients with SCLC, implicating a need to select patients harbouring BCL-2 amplification. Indeed, in common with other modes of targeted therapy, treating the right target population is likely to be a critical requirement for achieving clinically relevant activity when considered as single agents.

Recent analysis of genome-wide somatic copy number variations in cancer has revealed BCLX encoded by BCL2L2 and MCL-1, as the most frequently amplified genes in the cancer genome, and are encoded at 1q21.2 and 20q21 respectively (16). Where there is evidence of amplification, this appears to be associated with addiction, at least at the preclinical level. A proportion of NSCLCs harbour amplification at these loci, suggesting that addiction could be exploited. One novel approach has been recently reported. A search for transcriptional repressors of MCL1 (which has an exceedingly short protein half-life of around 30 minutes) identified anthracyclines as potent MCL-1 inhibitors (50). These compounds owe their proapoptotic activity to the transcriptional repression of MCL1, leading to its rapid downregulation at protein level. In the context of 1q21.2 amplification, this is associated with induction of apoptosis. This raises the intriguing question as to whether or not anthracyclines may exhibit particularly high activity in the context of 1q21.2 amplification in NSCLC, and deserved to be addressed in a clinical trial. High dose epirubicin has an associated response rate of around 25%, and 1q21.2 amplification occurs in around 25% of patients (36,51). Whether the majority of responders to epirubicin were also 1q21.2 amplified, is as yet, unknown.

Taken together, it appears that addiction to prosurvival BCL2 family proteins is restricted to subsets of lung cancers. These subsets may be identifiable through detection of somatic mutations involving amplification. Apoptotic agents targeting prosurvival BCL2 proteins are, when considered as monotherapy, are likely to be much like any other targeted agent, in that they may only exhibit useful efficacy in restricted subsets of cancers, perhaps

identifiable through individual copy number variations.

Death receptors

The apoptosis pathway can be directly activated through the ligation of cell surface receptors related to the tumour necrosis factor superfamily which include tumour necrosis factor related apoptosis inducing ligand (TRAIL) receptors (52,53). A direct consequence of receptor oligomerisation is the assembly of a cell surface signalling module (the death inducing signalling complex or DISC), which comprises homotypic domain interactions between receptor (TRAIL receptor 1 or 2), an adaptor (FADD), and an apical caspase (8 or 10). The proapoptotic activity of TRAIL ligands is selective for cancer versus normal cells (54). Recently, it has been shown that *in vivo*, disruption of tumour endothelial vasculature by TRAIL causes tumour regression (55). Agonistic antibody based activation of receptors for TRAIL have been explored in a series of recent phase II clinical trials in non-small cell lung cancer (56,57). Preclinical studies demonstrated promising synergy when combined with chemotherapy and other targeted agents (58-61). Unfortunately, predicted improvement in efficacy was not confirmed in unselected patients (62). Despite this, it has been found that TRAIL monotherapy is potentially very active in a small population of patients with NSCLC. For example, one patient with chemorefractory NSCLC exhibited a confirmed response lasting 96 weeks following the agonistic DR5 antibody (conatumumab, AMG-655) (63). This potentially reflects an underlying “hypersensitive” subgroup for which, there is at present, no validated predictive biomarker. TRAIL agonists are inhibited by the cellular FLICE like inhibitor protein (c-FLIP) which exhibits high expression in non-small cell lung cancer, and the ratio of FLIP to caspase 8 is a potential rheostat, regulating sensitivity to TRAIL receptor agonists. Similarly, O’glycosylation (64) and VDAC1 have been implicated as regulators of TRAIL sensitivity preclinically (65). What role these potential biomarkers have *in vivo*, if any, should be systematically investigated in future studies in order to maximize the likelihood of identifying a TRAIL receptor agonist sensitive population; something which clearly exists, albeit perhaps at low frequency.

Smac’ing lung cancer

During permeabilization of the mitochondrial outer

membrane, one of the apoptogenic factors released is the second mitochondria derived activator of apoptosis (SMAC) (20). Since its discovery, SMAC was shown to target inhibitor of apoptosis proteins, which constitutively suppress caspase activation and therefore the execution phase of apoptosis. The conserved tetrapeptide motif AVPI in SMAC interacts with the BIR domain of caspase 3, blocking its activation. Structure based analysis of this interaction led to a rational drug discovery effort to create so-called smac mimetics (66,67). This class of pharmacology however was shown to uncover a programmed necrosis pathway (68,69). In an inflammatory microenvironment, cytokine activation of TNF receptors leads to the assembly of a so called type 1 complex, which is prosurvival, and signals to caspase 8 through NF kappa beta. This signalling is dependent upon bound cIAP1 and cIAP2. SMAC or its mimetics interact with cIAP1/cIAP2 leading to their rapid ubiquitination and degradation. The consequence is the recruitment of TNF receptor with caspase 8 into complex II, comprising RIP kinase which leads to necrotic death of the cell. This death signalling is driven by TNF receptor activation; as such, the conversion of a survival pathway, to a death signalling pathway following IAP degradation, effectively exploits the tumour microenvironment and so constitute a “death switch”.

SMAC mimetics are at the earliest stage of development with respect to “apoptotic agents” and are currently under phase 1 evaluation in the clinic (70). At present, there are, as yet no defined molecular biomarkers of clinical sensitivity, however it is clear from preclinical studies that autocrine TNF-alpha activation facilitates the synergistic interaction between SMAC mimetic and chemotherapy (71). Accordingly, there is an expectation that this class of agent might be most effective in highly inflammatory cancers.

Systematic approaches for personalising apoptotic agents

An initiative entitled the genomics of drug sensitivity established as a collaboration between the Wellcome Sanger Institute in the UK and Massachusetts General Hospital/Harvard, in the USA, provides a potentially high throughput platform for identifying genetic biomarkers of sensitivity and/or resistance, that might aid clinical development of apoptotic agents (72,73). Using over 1,000 genetically defined cell lines, a candidate drug is screened for sensitivity. The correlation between sensitivity measured by IC50 and genetic mutations are determined. As such,

this provides a remarkably powerful tool for hypothesis generation, particularly around hitherto unanticipated but statistically robust drug-gene associations. For example, for ABT-263, the CML driver oncogene *bcr-abl* is highly correlated with *in vitro* activity (72).

Summary

Efficient induction of apoptosis is a prerequisite for effective disease control in the management of lung cancer, exemplified by receptor tyrosine kinase inhibitor efficacy in EGFR and EML4-ALK mutated NSCLC. Decades after the discovery of the core apoptosis signalling pathways, apoptotic agents have finally been developed with potent on-target activity. Population based genetic heterogeneity of lung cancer is now an accepted reality that has underpinned successful stratified therapy. Despite this, development of apoptotic agents has been predominantly conducted in unselected populations. The challenge moving forward will be understand how best to target these drugs using molecular biomarkers, so as to maximize patient benefit in selected subgroups.

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