JOE DOUPE LECTURE

Chemical biology – understanding biology and advancing therapy

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Abstract

Chemical biology is a new academic discipline whose goals are to understand biological systems using chemistry and chemical compounds. In addition to serving as important biological probes, some of these small molecules could be useful therapeutically. This review will discuss how advances in chemical biology by academics can aid in the discovery of new anti-cancer agents. Specifically, novel molecules can be useful tools to validate a potential target in a disease site and help provide the rationale for a clinical trial with a related molecule developed by industry. These novel molecules can also be developed for clinical use, although obstacles to this approach are recognized. Finally, this review discusses the opportunities to identify off-patent drugs with previously unrecognized anti-cancer activity and how the prior data on the drug would permit it to be rapidly repurposed.

Chemical biology – an emerging academic discipline

Chemical Biology is an emerging academic discipline whose goal is to understand biological systems at the molecular level using chemistry and chemical compounds, and thereby advance both the fields of biology and chemistry. Until the last five to ten years, the application of chemistry to biological systems was almost exclusively a domain of the pharmaceutical industry. However in the last several years, chemical biology has emerged as a prominent discipline in aca-

demie institutions. Several factors, in my opinion, have facilitated the emergence of chemical biology as an academic field of study. First, the sequencing of the human genome has uncovered many potential therapeutic targets that can be interrogated using a chemical biology approach. Second, there is a greater emphasis on interdisciplinary collaborations in scientific research, and chemical biology is a field based on cross-disciplinary science. Third, the cost of the robotics and libraries that are required for chemical biology projects has declined due to technical advances. As a result, major academic centres can now establish the infrastructure to support chemical biology research. Finally, over the last 5-10 years, funding organizations have actively supported chemical biology. For example, the National Institutes of Health (NIH) Road Map identified chemical biology as one of the priority areas for NIH research and funding. In addition, funding agencies such as the Ontario Institute for Cancer Research (OICR) have established granting competitions to fund chemical biology projects and high throughput screening initiatives in academic centers. Thus, the convergence of these factors has sparked the field of chemical biology and has attracted scientists' worldwide. For example, graduate training programs in Chemical Biology have emerged at leading academic institutions. In addition, the Nature publishing group

launched the journal “Nature Chemical Biology” in 2005.

My research interests have focused on the identification of chemical compounds to serve as probes to understand the molecular basis of cell death pathways in malignancy. As a Clinician/Scientist, an additional goal of my team has been to advance new therapies from the bench to the bedside. This review will highlight some of the progress we are making in developing novel therapeutics and how we are using these compounds to understand the biology of malignancy. Broadly, my team has taken two approaches to developing novel therapeutics. In the first approach, we have identified novel chemical compounds and used them as tools to validate a therapeutic target for a given disease site. In the second approach, we have worked to move therapeutics directly from our lab to clinical trial. In this regard, we have focused on identifying off patent drugs with previously unrecognized anti-cancer activity.

Novel chemical compounds – from bench to bedside

The identification and characterization of small molecule XIAP inhibitors highlights some of our efforts to develop novel chemical compounds and use them as tools to validate a therapeutic target. XIAP is a member of the IAP family of apoptotic proteins that inhibits the downstream portion of the apoptosis pathway. Specifically, XIAP binds and inhibits active caspases 3/7 and 9, but not caspases 1, 6, 8 or 10. By inhibiting the downstream effector caspases, such as caspases 3/7, XIAP blocks apoptotic signals generated by a diverse array of stimuli. Malignant cells frequently over-express XIAP and, in selected malignancies, over-expression of XIAP is associated with a worse clinical outcome.¹ Thus, inhibitors of XIAP would be useful probes to better understand the role of this apoptotic regulator in both normal and malignant cells. In addition XIAP inhibitors would be leads for a new class of therapeutic agents.

Therefore, we sought to identify small molecule XIAP inhibitors and devised an enzymatic assay to screen for small molecules that derepress XIAP's ability to inhibit caspase 3.² In our enzymatic assay, recombinant XIAP protein was combined with recombinant active caspase 3 in order to inhibit approximately 75% of the active caspase activity. With this assay, we screened a combinatorial library of chemical compounds for molecules that would inhibit XIAP as evidenced by an increased ability of the recombinant caspase to cleave a fluorogenic substrate. From this enzymatic screen, we identified a series of XIAP inhibitors including compounds based on the polyphenylurea pharmacophore such as 1396-12, 1396-22 and 1396-34. We demonstrated that these small molecules bound XIAP at its BIR2 domain³, which is the region of XIAP responsible for binding and inhibiting active caspase 3.⁴ In contrast, these molecules did not bind the BIR3 domain of XIAP³, which is responsible for binding and inhibiting caspase 9.^{4,5} In addition, we demonstrated that these compounds rapidly induce apoptosis in malignant cells through a mechanism consistent with XIAP inhibition.²

Subsequently, we used these compounds as tools to investigate the effects of inhibiting XIAP in leukemia cell lines and primary patient samples as well as normal hematopoietic cells.⁶ We demonstrated that the polyphenylurea XIAP inhibitors induced apoptosis in leukemia cell lines and primary acute myeloid leukemia (AML) patient samples with a LD₅₀ in the low micromolar range, but were not toxic to normal hematopoietic cells in short-term cytotoxicity assays. Among the primary AML samples, response to the XIAP inhibitors correlated with levels of intracellular XIAP protein. Patient samples with low to absent levels of XIAP protein were resistant to cell death after treatment in vitro with the XIAP inhibitors. In contrast, XIAP inhibitors were most active in vitro in patient samples with high levels of XIAP protein. As such, we hypothesize that there are two groups of patients with AML. In the first group, comprising approximately 40% of AML patients, XIAP levels are

FIGURE 1. XIAP inhibits apoptosis in response to diverse stimuli. XIAP binds and inhibits active caspase-3/7 and -9. As such it blocks apoptosis in response to a diverse range of stimuli including stimuli that activate the mitochondrial, death receptor, or endoplasmic reticulum-mediated pathways of caspase activation.

low and do not contribute to the disease pathogenesis. In this group of patients, inhibiting XIAP has no effect on cell death, due to the lack of target. In contrast, in 60% of AML patients, XIAP protein is increased compared to normal hematopoietic cells and contributes to disease pathogenesis by blocking active caspases. In these patients, derepressing XIAP leads to cell death. Thus, using a chemical biology approach, we have furthered the understanding of the role of XIAP in both malignant and normal cells. Furthermore it has helped validate XIAP as a therapeutic target in AML.

Currently, the polyphenylurea XIAP inhibitors are moving forward in clinical development and second generation compounds are being evaluated in pharmacokinetic and toxicology studies. We are encouraged about the potential utility of these agents as novel anti-cancer therapies, but advancing new chemical compounds like the polyphenylurea inhibitors into clinical trial is a slow process that can take up to ten years as their toxicology, pharmacokinetics, stability, etc, need to be investigated and optimized. In the interim, having validated XIAP as a therapeutic target in AML, we have partnered with Aegera Therapeutics to

initiate a clinical trial using their XIAP antisense oligonucleotides. Antisense oligonucleotides directed against XIAP bind intracellular XIAP mRNA and promote the degradation of XIAP mRNA by recruiting the enzyme RNAase H that selectively degrades the sense strand leaving the antisense strand intact. Based in part on our data as well as the findings that XIAP antisense oligonucleotids or siRNA induce cell death, delay tumour growth, and sensitize malignant cells to chemotherapy⁷⁻¹¹, we initiated a phase I/II clinical trial of XIAP antisense in combination of reinduction chemotherapy for patients with AML. In this study, patients with relapsed or refractory AML receive reinduction chemotherapy with idarubicin and high dose cytarabine in combination with XIAP antisense oligonucleotides. To date, the antisense has proved safe without significant toxicity. The phase I portion of the study is closed and we are completing the phase II portion where efficacy is being assessed. In the context of this trial, we are also evaluating the ability of the antisense to knock-down its target and induce apoptosis.

Off-patent drugs with previously unrecognized anti-cancer activity – a rapid route to clinical trial

As noted above, developing a novel chemical compound into a drug suitable for clinical trial is a slow process that can take upwards of several years. Furthermore, most novel chemical compounds that display activity in the laboratory never reach the clinic due to difficulties with issues such as toxicity, solubility, or stability. Therefore, to overcome these problems and rapidly advance new compounds from the bench to the bedside, we have attempted to identify off-patent drugs with previously unrecognized anti-cancer activity. By relying on their prior safety, pharmacokinetics, solubility and stability data, we can rapidly repurpose these drugs and advance them to clinical trial for a new anti-cancer indication within as little as two years from discovery.

The repurposing of thalidomide as a novel anti-myeloma therapy is an example of the potential success of this drug recycling strategy. Thalidomide was initially developed as a treatment for nausea in pregnancy, but withdrawn from the market in 1961 due to teratogenicity. In 1999, thalidomide was reported to have activity in multiple myeloma and produced a response in 32% of patients with refractory disease.¹² Subsequently, thalidomide in combination with melphan and prednisone was shown to prolong survival of older patients when compared to melphan and prednisone alone.¹³ The success of thalidomide led to the development of the second generation analogue revlimid that also improves outcomes in patients with multiple myeloma as well as other hematologic malignancies such as myelodysplasia.^{14,15}

Our high throughput screen to identify inhibitors of cyclin-D2 transactivation is an example of our efforts to identify off-patent drugs with previously unrecognized anti-leukemia and anti-myeloma activity. We sought to identify inhibitors of cyclin-D2 transactivation, because D-cyclins are over-expressed in virtually all patients with myeloma and this over-expression contributes to the pathogenesis of this disease.¹⁶ Likewise, over-expression of D-cyclins is also associated with worse clinical outcome in patients with AML.¹⁷ Therefore, inhibitors of D-cyclin transactivation would be a marker of a potentially active anti-leukemia or anti-myeloma therapy. In addition, these molecules would be useful probes to better understand the regulation of D-cyclins in these malignancies. Using our automated assay, we screened over 5000 compounds from the LoPAC, Prestwick, and Spectrum libraries of off-patent drugs, natural products and chemicals for inhibitors of cyclin-D2 transactivation.¹⁸ From this screen, we identified several hits including the glucocorticoid family of compounds. Glucocorticoids such as dexamethasone inhibited cyclin-D2 transactivation and decreased levels of cyclin-D2 mRNA and protein. In subsequent studies, we demonstrated that dexamethasone down-regulated cyclin-D2 in myeloma cell lines by promot-

ing the ubiquitination and proteasomal degradation of the oncogene c-maf that drives expression of cyclin-D2. Dexamethasone increased c-maf ubiquitination by increasing expression of ubiquitin mRNA.¹⁸ Further work demonstrated that dexamethasone enhanced the binding of the SP1 transcription factor to the ubiquitin promoter without increasing levels of SP1 mRNA or protein.¹⁹ Thus, this chemical biology screen identified a novel mechanism of action for dexamethasone that may partly explain its known anti-myeloma activity. Furthermore, this screen provided new insights into the regulation of c-maf protein in myeloma cells and identified c-maf as the first protein whose proteasomal degradation is regulated by levels of the ubiquitin substrate. While these results improve the understanding of the biology of multiple myeloma, they, unfortunately, will not influence the therapy of this disease. Currently, steroids are a primary treatment of myeloma, so this work will neither encourage nor discourage the use of these drugs for this condition.

However, our screen also identified other D-cyclin inhibitors that down-regulate D-cyclin expression through mechanisms unrelated to c-maf. Specifically, we identified the cyproheptadine that was previously used for the treatment of migraines²⁰, anorexia²¹, and atopic dermatitis.²² We demonstrated that decreased D-cyclin expression in leukemia and myeloma cells.²³ In addition, cyproheptadine induced cell-death in myeloma and leukemia cells at low micromolar concentration and delayed tumour growth in mouse models of leukemia.²³ Mechanistically, cyproheptadine decreased expression of the transcription factor AP2 that drives D-cyclin transactivation. Moreover cyproheptadine inhibited D-cyclin expression and induced cell death through a mechanism unrelated to its known binding targets of the H1 and serotonin receptors.²³ Thus, cyproheptadine could represent a lead for a new anti-leukemia therapy. Alternatively, it could be directly repurposed for such an indication. However, it is unknown if anti-tumour effects of cyproheptadine can be obtained in humans after systemic administration of the drug.

Our screen for inhibitors of cyclin D2 transactivation also identified the natural product kinetin riboside that reduced levels of cyclin-D1 and D2 but not cyclin-D3 through a mechanism independent of c-maf.²⁴ Kinetin riboside induced cell-death in myeloma cell lines in primary patient samples, preferentially over a normal hematopoietic cells. Moreover kinetin riboside inhibited tumour growth in myeloma xenografts. Mechanistically, kinetin riboside upregulated the CREM transcription factor that regulates D-cyclin expression. Thus, kinetin riboside may also be a lead for a novel anti-myeloma therapy or itself could be repurposed for this new indication.

Finally, our screen identified the off-patent anti-parasitic agent Clioquinol. Clioquinol is a halogenated 8-hydroxyquinoline that was used in the 1950s-1970's as an oral anti-parasitic agent for the treatment and prevention of intestinal amoebiasis, but its mechanism of action as an anti-microbial was unknown. The drug was routinely administered orally at doses exceeding 2g per day over a period of weeks. However, in the 1970s the drug was withdrawn from the market as an oral agent due to an association with sub-acute myelo-optic neuropathy (SMON) in Japanese patients. SMON is a syndrome that involves sensory and motor disturbances in the lower limbs as well as visual changes²⁵ that are due to symmetrical demyelination of the lateral and posterior funiculi of the spinal cord, optic nerve, and peripheral nerves.²⁶ The majority of symptoms were reversible, but permanent disability has been reported in 1000 of the 10,000 total cases of SMON noted in Japan²⁷ over the two decades the drug was in use. In the Japanese patients with SMON, the side effects emerged after prolonged treatment, as the average cumulative dose of Clioquinol in the cases was 136 g.²⁶ Notably, these side effects occurred almost exclusively in Japanese patients and cases of SMON in patients outside of Japan are exceedingly rare and are essentially at the case report level.²⁸ In fact, before the withdrawal of Clioquinol from the market there were 10,000 cases in Japan and only 220 cases in the rest of the world despite its use for over

500 million patient days.²⁹ The explanation for the neurological side effects among the Japanese is unknown, but many of these cases may have been related to concomitant vitamin B12 deficiency.²⁹ Alternatively, the neurological side effects may have related to the different formulation of Clioquinol used in Japan or genetic susceptibility to this side effect among Japanese.^{25,29} While Clioquinol is no longer used as an oral agent, the drug itself is approved by Health Canada and the FDA and remains in use for the treatment of topical bacterial and fungal infections. Clioquinol is available in Canada in the topical preparations, Locacorten-Vioform Cream and Eardrops as well as Vioform Hydrocortisone Cream. In the last several years, Clioquinol has re-emerged as a potential therapy for Alzheimer's disease. The activity in Alzheimer's disease relates to the ability of Clioquinol to bind copper. Clioquinol binds copper and dissociates this metal from β -amyloid protein aggregates that have been associated with Alzheimer's disease.³⁰ Upon removal of copper, the aggregation of these proteins is reversed.³⁰ A phase II study of oral Clioquinol in patients with Alzheimer's disease reported that the drug was safe and improved the cognition and behaviour of their study patients.³¹

We demonstrated that Clioquinol induced cell death preferentially in leukemia and myeloma cell lines and primary patient samples preferentially over normal hematopoietic cells. Furthermore, in three mouse models of leukemia it delayed tumor growth without untoward toxicity. Mechanistically, Clioquinol inhibited the proteasome through a dual mechanism of action. It mobilized intra and extracellular copper stores and directed copper to the proteasome where the copper caused disruption of the proteasome structure and function. These results were consistent with previous reports describing Clioquinol as a copper-dependent proteasome inhibitor. However, in a previously undescribed mechanism of action, Clioquinol also inhibited the proteasome through a copper-independent mechanism of action.³² Thus, Clioquinol

differs from all other previously reported chemical proteasome inhibitors that only bind the active enzymatic sites and inhibit the proteasome competitively.

Given that the concentrations of Clioquinol required to produce an anti-leukemia and anti-myeloma effect appear to be achievable in patients after systemic administration, we are moving Clioquinol rapidly towards phase I clinical trial in patients with refractory hematologic malignancies. Through this study we will determine the optimal phase II dose and determine whether the drug can inhibit the proteasome in patients.

Conclusions

Small molecules that impact the cell death pathways in malignant cells are useful probes to understand this biological process. In addition, some of these compounds may be useful therapeutically and be leads for novel anti-cancer compounds. By identifying of-patent drugs with anti-cancer properties there is a possibility to repurpose these agents for this new indication and rapidly translate discoveries from the bench to the bedside.

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