RNA INTERFERENCE (RNAi): AN EFFECTIVE WAY TO DEVELOP RATIONAL COMBINATION THERAPIES WITH HYPOMETHYLATING AGENTS IN ACUTE LEUKAEMIAS AND MYELODYSPLASTIC SYNDROME

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ABSTRACT

Therapeutic progress in aggressive myeloid malignancies such as acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS) and advanced myeloproliferative neoplasm (MPNs) has been slow. This is partially due to the heterogeneity of the diseases and the lack of molecular understanding, which delays effective drug development. There will be potentially three rather effective avenues to arrive at novel molecular vulnerabilities that can be therapeutically exploited. First, is identifying disease-specific mutations or other genomic aberrations (i.e. translocations, aberrant methylation) that will result in targeting affected and closely associated genes. Second, is tracing disease evolution over time, clonal evolution by various approaches, many of which will include current genomic tools and assays. Third, a more unbiased, broad discovery approach either with small molecule screens or, in our opinion, by identification of specific molecular vulnerabilities by RNA interference (RNAi). RNAi is a mechanistically rather agnostic high-throughput approach to find essential targets, alone or in combination with commonly used anti-cancer agents. In this paper we will briefly summarise some ideas and early results of RNAi screens, with a focus on hypomethylating agents and how RNAi can identify rational combination therapies with 5-azacytidine that can be rapidly translated into the clinical setting.

Keywords: Small interfering RNA, RNAi, AML, MDS, MPN, hypomethylating agents, 5-azacytidine, decitabine.

INTRODUCTION

The most common type of acute leukaemia in adults is acute myeloid leukaemia (AML), which is one of the malignancies with the highest mortality rate.[1] For most AML patients, treatment and outcome has not changed for decades; and especially for patients with relapsed and refractory disease, options are dismal. This is particularly true for the many elderly AML patients, that often do not qualify, or are poor candidates for cytotoxic chemotherapy, and for whom allogeneic transplantation therapeutic options are limited.[2] In addition, the biology of elderly AML is very distinct compared to younger patients (i.e. <40 years) and therefore the response rates are much less in elderly AML patients than for younger patients.[3] In contrast, multiple new therapies and agents have been developed for chronic leukaemias,[4] and outcomes for paediatric/young adult lymphoid leukaemias have remarkably improved.[5] Therefore, cytotoxic regimens do not benefit most elderly patients, and lower intensity therapies are similarly effective in AML patients with an age greater than 65-70 years.[6] The development and approval of hypomethylating agents and Janus kinase 2 (JAK2) inhibitors for myelodysplastic syndrome (MDS)[7] and myeloproliferative neoplasm (MPNs)[8] respectively, has improved the outcome for these myeloid malignancies. However, advanced aggressive forms of MDS have a low survival rate of 0.4-0.8 years, similar to AML.[9] Therefore, novel treatments and combinations are an urgent clinical need in relapsed

and refractory acute leukaemias as well as *de novo* treatment of AML, both for elderly AML patients and for aggressive forms of MDS and MPNs.[10]

The hypomethylating agents 5-azacytidine and decitabine have shown response and survival rates that are almost as good as cytotoxic chemotherapies in older patients with AML and MDS.[6] Still there remains a large number of patients in the range of 25-50% who do not respond to 5-azacytidine and decitabine, even in the upfront treatment. Ultimately, most patients become resistant and progress while being treated on 5-azacytidine.[11] Therefore, clinical-translational research goals are needed to improve upfront response rates to 5-azacytidine and decitabine and to overcome initial or developing resistance to hypomethylating agents. This article will provide an overview and examples of using RNA interference (RNAi) and give examples of practical translation of RNAi-derived genomic knowledge into clinical development.

In AML disease biology there is, to date, no single underlying genetic event that may be exploited as a sole drug target.[12] Cytotoxic combination regimens are still standard therapy options for patients curable by chemotherapy or those likely proceeding to allogeneic transplant. Nevertheless, cytotoxic regimens have reached their therapeutic limit[13] and new targeted agents directed at specific genomic aberrations will need to be developed in order to improve therapy outcomes while minimising side-effects. In AML, MDS and MPNs, genetic aberrations in myeloid transcription factors, mutations in kinases, and inactivation of 'growth controlling' genes such as tumour suppressor genes (i.e. p53 or apoptosis regulators[14]) act together in malignant transformation. Complementary structural molecular aberrations, such as mutations or translocations, co-occur together and operate on the basis of altered, epigenetic transcription. This is supported by the increasing number of mutations in 'epigenetic' genes.[14] Accordingly, single agent 5-azacytidine, decitabine or kinase inhibitors alone have limited single agent clinical activity. Therefore, several oncogenic aberrations should be inhibited simultaneously to achieve better clinical responses. To date however, the essential targets to interfere with, alone or in combination with hypomethyalting agents, are unknown.

5-Azacytidine Combinations

5-azacytidine and decitabine affects methylation, mostly by reducing global methylation, while differential methylation may occur at tumour suppressor (less methylation) versus on oncogenes (higher levels of methylation).[15] However, the true underlying mechanism of action of 5-azacytidine and decitabine is still poorly understood. Despite this undefined mechanism, 5-azacytidine and decitabine have been investigated clinically with a number of other drugs. Several trial reports combining hypomethylating agents with histone-deacetyltransferase (HDAC) inhibitors have been published, for example suberoylanilide hydroxamic acid (SAHA), valproic acid, or entinostat. However, response rates were only modestly improved in combination. The overall response rates (ORR) (often defined as complete remission (CR) or CR with incomplete count recovery (CRi), partial remission (PR), and sometimes stable disease (SD)) range from ~25 to ~50%[16-18] depending on the study, with possibly higher ORR in newly diagnosed untreated patients of up to 67% as reported in one publication.[19]

Another frequently studied combination is combined 5-azacytidine and lenalidomide. A rationale is provided based on clinical grounds, as both have single-agent activity in AML and MDS and rather few overlapping side-effects, except possibly myelosuppression. A consideration to their known mechanism is that 5-azacytidine affects cycling cells and lenalidomide inhibits cell cycle progression. Hence, the reported studies tested different schedules including sequentially versus concurrent dosing, as well as slightly different lenalidomide doses. Furthermore, the patient population in the reported studies varied, which may explain some of the differences in the reported responses. Generally lenalidomide at 10 mg was the best tolerated dose with full dose 75 mg/m^2 5-azacytidine; some studies escalated lenalidomide to 50 mg daily during the first cycles, which was generally found to be too toxic when combined with 5-azacytidine.[20] 5-azacytidine-lenalidomide combinations were tested in higher risk MDS and in AML patients. The ORR for most studies across MDS and AML are roughly in the range of ~41-75%.[20-23] The highest response rate was a 44% CR rate in one MDS study; however, the median survival of 13.6 months in that study was lower than the 24.5 months in Aza-001 study,[24] the landmark study that tested single-agent 5-azacyditinde in MDS. In untreated elderly AML, a similarly high CR/CRi rate of 44% (n=7/16) was observed, however this study was small.

Comparable CR/CRi rates for single agent 5-azacytidine and decitabine in AML are around ~17-

18% with an ORR of up to 71% and 48% respectively for MDS or AML.[24,25]

Limited clinical evidence that lenalidomide may truly sensitise to 5-azacytidine comes from observations of three patients: all three patients received the 5-azacytidine-lenalidomide combination and at some point were taken off lenalidomide and continued on single-agent 5-azacytidine. At disease progression, lenalidomide was again added to 5-azacytidine and all three patients responded, suggesting that lenalidomide may indeed clinically sensitise to 5-azacytidine.[26] However, no firm conclusions can be drawn from these observations in these three patients, and larger studies regarding the potential of combining 5-azacytidine with lenalidomide or HDAC inhibitors are needed. In fact, a randomised Phase II trial is ongoing to compare single agent 5-azacytidine versus combination treatment of 5-azacytidine and lenalidomide (10 mg) or 5-azacytidine and SAHA in intermediate and high risk MDS. Similar studies are ongoing in the US and in Europe.

Novel Hypomethylating Drugs

Few next-generation hypomethylating agents are under development; for example, the decitabine pro-drug SGI-110, which is a dinucleotide of decitabine and deoxyguanosine. SGI-110 is cleaved into decitabine *in vivo*. This pharmacokinetic change is expected to prolong decitabine exposure and thereby increasing the effect on deeper hypomethylation. Data from a Phase I study of AML and MDS patients treated with SGI-110 were presented at the American Society of Hematology (ASH) 2012,[27] with an update at the annual meeting of the European Hematology Association (EHA) 2013.[28] Results showed that SGI-110 has a longer *in vivo* exposure than decitabine and achieves deeper de-hypomethylation. Complete and incomplete responses (CR/CRi/CRp) were seen, and haematological improvements were also observed.[27] However, with the expected heterogeneity of a Phase I patient population, it is too early to estimate response rates in comparison to the currently approved and commercially available drugs. A Phase II expansion cohort for newly diagnosed AML and MDS patients is ongoing and accruing patients.

RNAi Approach to Rational Combinations with Hypomethylating Agents

One of the main obstacles to developing rational 5-azacytidine or decitabine combinations is a lack of understanding of their mechanistic underpinnings. A broader, high-throughput target approach using RNAi screening may be able to avoid some of these limitations. An RNAi approach is rather 'agnostic', mechanistically unbiased a priori, and mostly limited by how many genes and which small interfering RNA (siRNA) libraries are included in the initial RNAi screens.

In brief, RNAi is a naturally occurring phenomenon[29] in many eukaryotes, including humans, arising from short RNA molecules that complementary bind specific messenger RNA (mRNA) molecules transcribed in cells. Through various intra-cellular processes, ultimately mRNA is degraded and not translated into proteins, which amounts to silencing or inhibition of a specific gene. Experimentally this natural occurring phenomenon is exploited by the design of synthetic siRNA molecules.[30] Libraries covering each human gene have been synthesised and are commercially available. These short RNAi molecules are transfected into leukaemia cells *in vitro* under concurrent treatment with, for example, 5-azacytidine.[31,32] With this approach, hundreds to thousands of genes can be inhibited individually at the same time and assessed if their inhibition augments the anti-leukaemic activity of anti-cancer drugs. A detailed description of the siRNA platform that our laboratory uses was recently published for a Cytarabine siRNA kinome screen.[33]

Up to now, only few large RNAi screens have been presented in leukaemia cells. Many of these tested inhibition of genes alone without addition of a drug. These screens can identify synthetic lethal interactions when a specific gene is inhibited.[34] One of the first siRNA screens assessed the tyrosine kinome and few other selected genes (altogether >100 genes) in AML cells.[35] Specific kinases were found that inhibited leukaemia cell growth. The same group extended their findings experimentally into patients to attempt to find functional relevant targets in patients' leukaemia cells.[36] Another landmark study utilised an shRNA (viral/vector based RNAi molecules) approach to assess 40-45 genes situated on the commonly deleted regions (CDA) of chromosome 5 in MDS. The gene RPS14 was found as a key regulator of disturbed erythropoiesis in 5q deleted MDS.[37] This study inspired the RNAi translational research field and contributed to understanding the so-called 5q MDS Syndrome, although it has not led to concrete therapeutic approaches.

Only few RNAi sensitiser screens, i.e. treating leukaemia cells with a drug and inhibiting many genes with the siRNA library, have been conducted.

An all trans-retinoic acid (ATRA) sensitiser screen has been published,[38] without translational application to date.

So far, our laboratory has presented the only high-throughput 5-azacytidine sensitiser screen in leukaemias.[39] We tested 861 genes individually, including the entire human kinome (that is, all known kinase genes) to determine if any of these genes, when inhibited by siRNA, would enhance the activity of 5-azacytidine. Surprisingly, kinases did not potentiate the activity of 5-azacytidine. However, in these screens we found the B-cell lymphoma 2 (BCL-2) family proteins as important sensitisers to 5-azacytidine. We subsequently used drugs in a clinical development that targeted B-cell lymphoma-extra-large (BCL-XL)[40] or BCL-2 respectively[41,42] and demonstrated that ABT-737 is synergistic with 5-azacytidine *in vitro* as well as in short term culture of samples from AML, MDS and MPN patients. While targeting BCL-XL or other anti-apoptotic molecules may be an applicable concept for cytotoxic therapies, the selectivity of sensitisation and lack of kinase targets identified in our assays from a large gene set, has important clinical implications for the selection of an agent to be combined with 5-azacytidine in clinical trials.

Recently, we conducted a similar siRNA screen of 289 genes on CDR of chromosome 5 and 7 in combination with 5-azacytidine, and identified a pathway that is potentially targetable, with validation studies currently ongoing. A clinical trial with yet another novel drug is in development based on the RNAi results (R. Tibes, personal communication).

These examples highlight an exciting potential of RNAi screens to yield high-profile targets that can be further evaluated as to their potential value for clinical translation into novel combinations. Given the high-throughput nature of RNAi, more targets are discovered than drugs are available and the rate-limiting step of translating RNAi findings into the clinic is the access and availability of drugs that correspond to the identified targets. Consequently, RNAi screens are increasingly conducted with parallel small molecule compound screens to increase the chances of discovering novel agents for clinical application.

One such example is the identification of BRD4 as a vulnerable gene in leukaemia cells. BRD4 regulates Myc expression via epigenetic mechanism.[43,44] Subsequently the agent JQ1 was discovered inhibiting BRD4 and thus epigenetic regulation of Myc.

Finally, RNAi screens can be used in an isogenic cell line or mutation context, i.e. a recent paper used FLT-3 mutated cells and performed a whole genome RNAi screen to find synthetic lethal targets in a FLT-3 mutational background and identify pathway concepts.[45]

CONCLUSION

In conclusion, the treatment and outcome of patients with advanced stages of AML, MDS and MPNs needs to be improved and new rational combination regimens developed. For drugs with a yet unclear mechanism of action (i.e. hypomethylating agents) and drugs that act rather non-specific, i.e. cytoroxics, an RNAi approach offers an economical, rapid way to discover genes that may modify the activity of known cancer drugs like 5-azacytidine. Novel molecular interactions can be identified, and if agents that target the respective genes hit from RNAi screens are available, these can be evaluated rapidly as to their potential for translation into rational combination clinical studies.

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