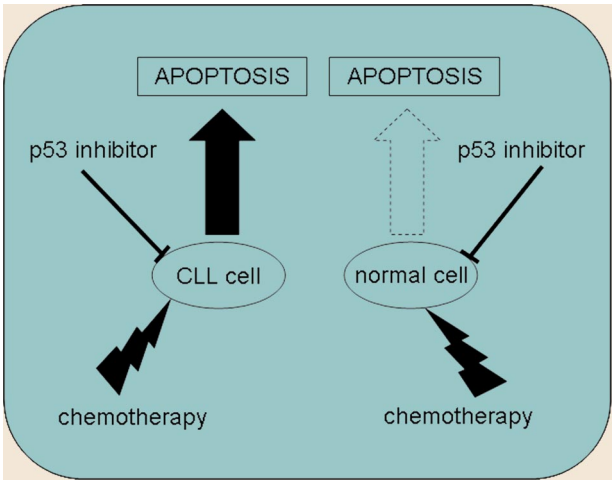

The combination of chemotherapy with p53 inhibitors enhanced CLL-cell apoptosis. In contrast, normal T cells were resistant to this combination of agents.

result is somewhat counterintuitive, but the authors conclude that the net effect of p53-induced transcription is the blockade of a transcription-independent apoptotic pathway. Therefore, selective inhibition of p53-mediated transcription by pifithrin α results in increased cell killing.

These results are intriguing but defy simple interpretation. For instance, it is not clear whether the effects of pifithrin α are truly the consequence of blocked transcription. It may be tempting to simplistically consider that pifithrin α blocks the transcription of the ubiquitin ligase MDM2, thereby stabilizing p53 and preventing its degradation.[3] Indeed, this paper presents evidence for pifithrin α–mediated inhibition of MDM2. However, p53 is capable of inducing/repressing hundreds of genes, so a painstaking dissection of global gene expression profiles induced by pifithrin α in CLL would seem necessary. This approach may yield a list of "likely suspects" that can be specifically inhibited using the gene silencing methodologies that are now available for the manipulation of CLL cells. Furthermore, a number of alternative explanations beyond transcriptional repression are possible. Pifithrin α may simply prevent p53 nuclear import, thereby sequestering it in the cytoplasm where it is free to interact with Bcl-2 family proteins. In this regard, the coimmunoprecipitation of Bcl-2 and p53 from CLL cell extracts presented here is intriguing. Can cytoplasmic p53 displace Bax from a Bcl-2/Bax complex, thereby triggering the Bax conformational change observed in this study? Alternatively, it has recently been suggested that p53 can even act directly on the mitochondrial permeability transition pore complex in order to induce apoptosis.[4] All of these possibilities can be tested experimentally, and so it seems inevitable that further revelations will be forthcoming in the near future.

This work has raised a number of very interesting biologic questions, but the key clinical question is, can we exploit p53 transcription blockers to augment current chemotherapeutics? The answer at the moment is a guarded "maybe." Promisingly, pifithrin α has been shown to be relatively nontoxic to normal tissue in mouse models,[5] and this present study shows little evidence of pifithrin α–mediated apoptosis in normal human T cells. However, additional studies involving the coadministration of standard chemo-therapies are clearly warranted. Another concern is that this strategy may only be effective when the CLL cells have a functional p53 pathway. p53 dysfunction is much more prevalent in advanced-stage and drug-resistant patients, so the clinical utility of this approach may be limited in this context.

Despite the unresolved questions and the probable clinical caveats, this paper provides fascinating new insights into the biological machinery of CLL cells. It seems likely that we will be able to exploit this new knowledge in the future to develop more effective treatments for this common but as yet incurable disease.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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NEOPLASIA

Comment on Pepper et al, page 3807

Mcl-1: the 1 in CLL

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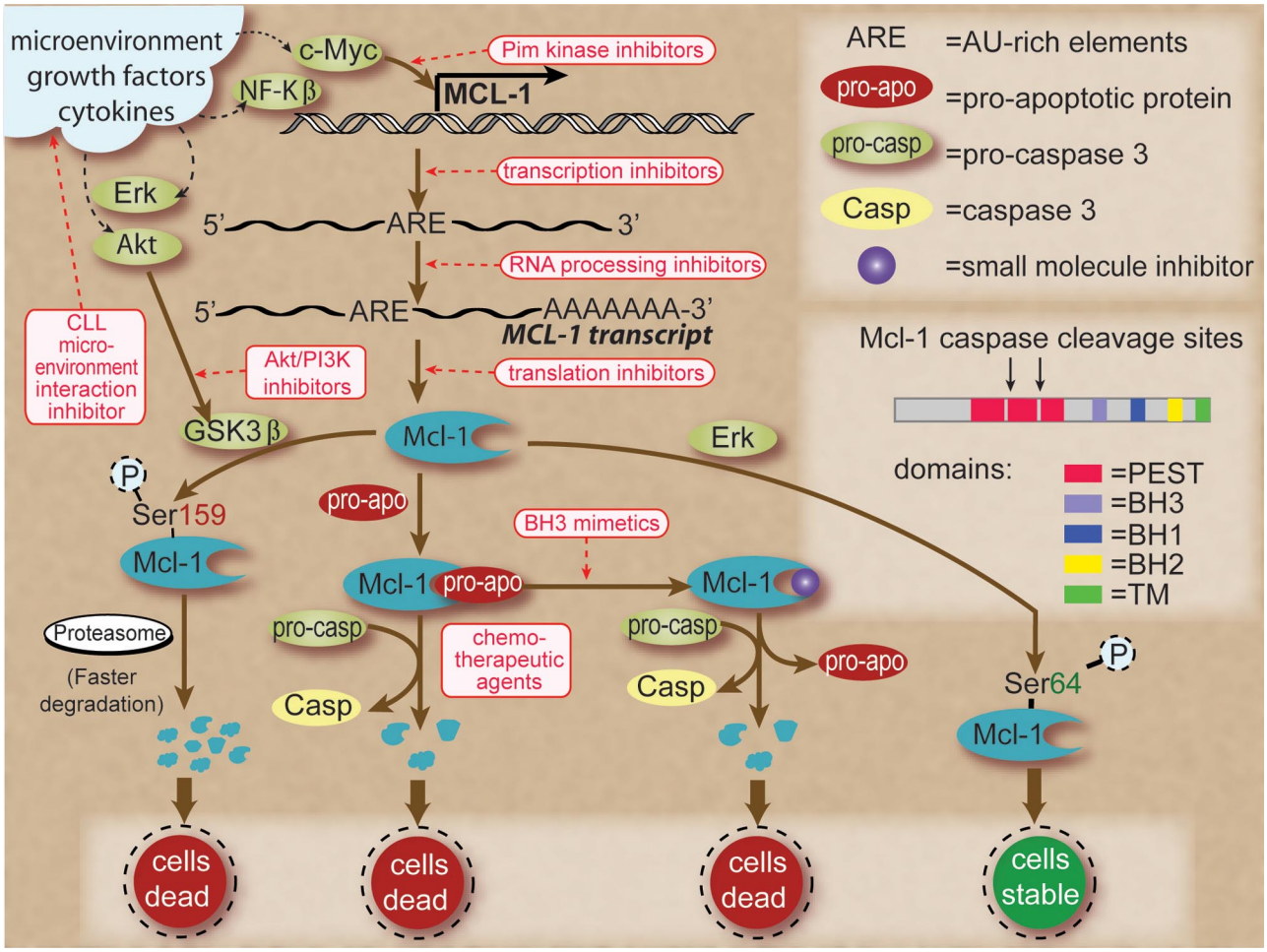
Although Mcl-1 has been established as a survival and maintenance protein during in vitro incubations of primary CLL cells, in this issue of *Blood*, Pepper and colleagues further demonstrate the association of Mcl-1 with other prognostic factors and the role of this antiapoptotic protein in disease progression and outcome for patients with CLL.

Chronic lymphocytic leukemia (CLL) is an indolent leukemia that is characterized by a relentless accumulation of mature lymphocytes with typical B-cell markers. Leukemic lymphocytes that are replicationally quiescent accumulate both in the bone marrow and the peripheral blood due to intrinsic defects in their apoptotic machinery and/or dysregulated production of survival signals from their Microenvironment. A balance between the anti- and proapoptotic proteins maintains the rheostat of B cells and all hematopoietic cells. While several members of antiapoptotic and proapoptotic Bcl-2 family have been identified, Mcl-1 surfaces as the most significant antiapoptotic protein associated with normal as well as malignant B lymphocytes.

Mcl-1 is essential during lymphoid development and maintenance of mature T and B lymphocytes[1], and the expression level of antiapoptotic proteins in normal and malignant lymphocytes is in concordance with its role in survival.[2] High levels of Mcl-1 and Bcl-2 mRNA and protein have been found in CLL, which are inversely correlated with in



Schema to show transcription, translation, and posttranslation modifications that lead to production and maintenance of Mcl-1 proteins in normal and/or malignant B cells and therapeutic strategies (shown in red rectangles and red text) to interfere with these processes. Signals from microenvironment, growth factors, and cytokines are shown in black dashed lines as they result in increased expression of transcription factors and survival factors in CLL cells. Small molecule agents that interfere with microenvironment and CLL cell interactions reduce survival signals. Myc-driven transcription could be inhibited by Pim kinase inhibitors. The adenylate/uridylate-rich elements in the 3' untranslated region of Mcl-1 transcripts target them for rapid degradation. Global transcription inhibitors, such as flavopiridol or polyadenylation inhibitors, work to reduce Mcl-1 transcripts due to their fast turnover. Pharmacologic agents that shut down protein synthesis result in lower Mcl-1 protein levels due to the short half-life of this protein. Phosphorylation of Mcl-1 on Ser159 by activated GSK3β (unphosphorylated form) results in faster degradation of Mcl-1 through proteasomal pathway. However, GSK3β could be phosphorylated and inactivated by Akt. Hence, Akt inhibition is a therapeutic strategy. Mcl-1 sequesters proapoptotic proteins, and the latter could be released by use of BH3 mimetics, leading to cell death and Mcl-1 degradation. Similarly, chemotherapeutic agents activate caspases that could cleave Mcl-1. Mcl-1 is phosphorylated on Ser64 by Erk, resulting in stabilization of Mcl-1 protein and cell survival. Inset shows the structure of Mcl-1 protein with BH1, 2, and 3 domains, transmembrane domain, and PEST sequences where caspase cleavage sites are located. Some of these pathways are recognized only in normal B cells and are of unknown relevance in CLL B lymphocytes.

vitro response to chemotherapeutic agents or with the failure of CLL patients to respond to fludarabine therapy.[3] Conversely, down-regulation of Mcl-1 protein expression by antisense oligonucleotides or through indirect Mcl-1 transcription and translation inhibitors results in cell death during in vitro culture or in vivo therapy. In addition, overexpression of Mcl-1 prolongs the survival of CLL cells exposed to a variety of apoptosis-inducing stimuli.[2] These key pieces of evidence establish Mcl-1 as a critical survival factor for CLL.

In this issue of *Blood*, Pepper et al use primary leukemia cells from a cohort of 185 patients with CLL to determine an association between antiapoptotic proteins and other prognostic parameters. To circumvent the qualitative nature of immunoblot analysis, flow cytometry was used to measure expression of antiapoptotic and proapoptotic proteins in a quantitative fashion. There is an expected heterogeneity among samples, yet the data beautifully demonstrate a relationship between Mcl-1 protein expression and other prognostic markers, such as stage of the disease, IgV_H mutation status, ZAP-70 positivity, and CD38 expression. The study begs for analysis of other antiapoptotic and proapoptotic molecules, although they have included Bcl-2 and Bax. We also need to appreciate that quantitation of protein levels in 185 primary samples is a phenomenal effort. They also confirm association between these proteins and in vitro sensitivity to fludarabine, however, this was not a direct relationship; the correlation coefficients (r^2 values) are not strong. Mcl-1 expression was also prognostic in overall survival of these patients and time to first treatment; the latter is under debate as there is not a consensus regarding the optimal time to treat CLL. Since the majority (70%) of the patients were of the Binet stage A, similar relationships were observed in this group also. Questions regarding a possible relationship between endogenous Mcl-1 levels and cytoreduction during therapy still remain unanswered. Nonetheless, the study provides new knowledge regarding the role of Mcl-1 in CLL patients,

something that was previously assumed but now is substantiated by data and statistical analyses.

So how can we take advantage of this knowledge? Since specific genetic targets are not defined in CLL, Mcl-1 seems to be an appropriate biomolecule to therapeutically manipulate. As shown in the figure, Mcl-1 protein production and maintenance are dependent on several pathways. At the apical level, the microenvironment provides factors that dramatically increase this protein in CLL cells.[4] Hence, a strategy that interferes with interaction of microenvironment and CLL cells is a logical approach. Production of Mcl-1 through these signals is carried via increased transcription of the *MCL-1* gene. Transcription and polyadenylation inhibition, albeit not selective, is an approach that works because of AU-rich elements in the transcript of Mcl-1, which leads to its rapid turnover.[5] The N-terminal region of Mcl-1 protein contains 2 PEST domains that are rich in proline, glutamic acid, serine, and threonine residues, resulting in a short half-life of the protein[5] and making translation inhibition and rapid degradation of endogenous Mcl-1 via proteasome pathway a viable option to reduce the protein level.[6] Mcl-1 is also essential during early lymphoid development[1] and is abundantly expressed in the germinal center B-cell compartment. Pim kinase and Akt-PI3–kinase pathways and down-stream of BLyS have been identified to maintain the Mcl-1 levels in B cells.[7] The roles of these pathways and consequence of their perturbations need to be investigated in malignant lymphocytes. Similarly, work is needed on posttranslational modification leading to increased or decreased half-life of Mcl-1 protein. Finally, and probably most intriguingly, small molecule antagonists of Mcl-1 protein that bind to the BH3 domain releasing proapoptotic proteins provide a new avenue of research and therapeutics.[2,8]

Conflict-of-interest disclosure: The authors declare no competing financial interests. ■

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NEOPLASIA

Comment on Liu et al, page 3835

Why is CLL refractory to bortezomib?

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In this issue of *Blood*, Liu and colleagues present evidence that flavonoids present in plasma may compromise the ability of the proteasome inhibitor bortezomib to induce apoptosis of CLL cells.

Bortezomib is a dipeptide boronate proteasome inhibitor which induces apoptosis of chronic lymphocytic leukemia (CLL) cells in vitro.[1] However, treatment of CLL patients with this agent generated no objective responses.[2] In this issue of *Blood*, Liu et al present evidence indicating that the discrepancy between the in vitro and in vivo observations may be accounted for by a chemical reaction between bortezomib and plasma components. Initial observations showed that while CLL cells cultured in media containing 10% fetal calf serum were induced to apoptosis by bortezomib, this action was dramatically compromised in the presence of 50% fresh human plasma. Further studies showed that the dietary flavonoid quercetin, which is present in plasma, blocked CLL cell killing by bortezomib. This action was confirmed by carrying out multiple cellular and molecular assays which showed that bortezomib-induced CLL cell killing occurs via the classic mitochondria-dependent intrinsic apoptotic pathway and that these mechanisms are compromised by quercetin.

The blocking effect of flavonoids is attributable to a direct chemical reaction between the boronate moiety of bortezomib and adjacent hydroxyl groups present on the B ring of some, but not all, flavonoids. First, quercetin and myricetin, which contain adjacent hydroxyls, effectively blocked bortezomib's cytotoxic action, while kaempferol and apigenin, which do not contain adjacent hydroxyls, failed to do so. Second, cell killing by the proteasome inhibitors MG-132 and lactacystin, proteasome inhibitors which do not possess a boronate group, was unaffected by flavonoids, which compromised apoptosis induction by the boronate compounds, bortezomib and MG-262. Finally, data obtained using Raman spectroscopy were consistent with a direct chemical reaction between quercetin and bortezomib.

Liu et al also show that boric acid reacts with quercetin and abolishes its inhibitory action on bortezomib-induced apoptosis. Boric acid also abrogated the protective action of plasma, suggesting that the ability of plasma to compromise CLL cell killing by bortezomib is indeed attributable to flavonoids. However, questions remain concerning the actual levels of flavonoid species present in plasma. While plasma levels of quercetin are insufficient on their own to account for the quenching effect, other flavonoid species are present in plasma. It would therefore be of interest to identify and quantify those species which are reactive with bortezomib. The concentration issue is particularly relevant since the actions of quercetin are significantly dose-dependent: While 20 μM quercetin effectively blocked killing of CLL cells by bortezomib, concentrations of 40 μM or greater actually induced apoptosis, an action that may be explained by the apparently paradoxical observation that quercetin itself may, like bortezomib, inhibit the β5 subunit of the proteasome.[3]

The studies here raise a further question: If plasma flavonoids effectively neutralize the cytotoxic actions of bortezomib on CLL cells, how is the observed effectiveness of this agent in multiple myeloma[4] explained? The authors suggest that interactions between flavonoids and CLL cells on the one hand and myeloma