efficiently while not interfering with the expression of other genes in the genome.

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

REFERENCES


THROMBOSIS AND HEMOSTASIS

Comment on Chen et al, page 1974

Vitamin K epoxide reductase: moving closer to nature

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In this issue of Blood, Chen et al have used a novel cell-based assay to evaluate the antagonist effects of 4 clinically prescribed vitamin K antagonists (VKAs) on the activity of vitamin K epoxide reductase (VKOR) and its VKA-resistant mutants in their natural cellular environment.

The first VKA to be used clinically was bishydroxycoumarin (dicumarol), the compound identified by Link’s group in Wisconsin as the anticoagulant present in spoiled sweet clover that was the cause of an often lethal hemorrhagic disease in cattle. Clinical trials of dicumarol as an oral anticoagulant began in 1940 and, within 2 years, more than 100 related 3-substituted, 4-hydroxycoumarins had been synthesized by Link’s laboratory for biochemical and clinical appraisal. One of these compounds was warfarin which was originally thought to be too potent for clinical use but which Link thought would make an ideal rodenticide. Indeed, after its introduction in 1948, warfarin revolutionized rodent control and, after a failed suicide attempt by a naval officer, equally revolutionized oral anticoagulant therapy.

Today, warfarin is still the most commonly prescribed oral VKA, but some countries use the related 4-hydroxycoumarins acenocoumarol and phenprocoumon or the 1,3-indandione derivative fluindione. All these VKAs were introduced into clinical practice without much knowledge of their molecular mechanism of action. It was not until the 1970s that the enzyme VKOR was identified as the target enzyme which, when inhibited, blocks the hepatic synthesis of active (γ-carboxylated) vitamin K-dependent coagulation factors (see figure). Because of the indirect mechanism of VKA action, a single blocking dose of warfarin does not produce the maximum hypoprothrombinemic response until 2 to 4 days after administration. This delayed pharmacodynamic response together with variable pharmacokinetics makes it difficult to compare the in vivo potency of different VKAs in humans.

The 2 major enzymes of the vitamin K cycle, γ-glutamyl carboxylase (GGCX) and VKOR, are integral membrane proteins that reside in the endoplasmic reticulum of cells and require cofactor forms of vitamin K that are themselves hydrophobic. For VKOR, the difficulty in evaluating structure-function relationships is that traditional functional assays are carried out in detergent-solubilized microsomal fractions using the nonphysiological reductant dithiothreitol (DTT) as a substitute for the unknown physiological reductants. The inherent artificiality of DTT-driven assays often provides an inaccurate picture of VKOR activity in vivo. After using such assays, only about one-third of missense VKOR variants defined as resistant to first-generation VKAs in humans or rodents showed the expected degree of resistance.

The study of GGCX and VKOR has been significantly advanced by the introduction of cell-based reporter assays in conjunction with gene-editing techniques. The principle is to express a chimeric vitamin K-dependent reporter protein in mammalian cells in which the reporter γ-carboxylation status can be readily measured by an enzyme-linked immunosorbent assay. In this way, one can knock out the specific gene of interest (GGCX or VKOR) in the reporter cell line and introduce a recombinant gene expressing the desired mutant in its place.

The first objective of Chen et al was to make a direct comparison of the inhibitory potency of 4 clinical VKAs as measured by the efficiency of carboxylation of the chimeric reporter protein and the half-maximal inhibitory concentrations of each VKA. The standing finding was that the efficacy of inhibition of wild-type VKOR followed the order of acenocoumarol > phenprocoumon > warfarin > fluindione, with the efficacy of acenocoumarol being fivefold to eightfold higher than that of the other VKAs. These observations were then extended to the determination of the carboxylation efficiency in the same cell-based assay when the cells expressed each of the 27 clinically identified VKA-resistant mutations of VKOR. This sub-study determined that most but not all of these VKOR mutations showed a degree of resistance consistent with the clinical picture. Acenocoumarol, which had already been shown to be the most potent inhibitor of VKOR-dependent
Enzyme activities of the vitamin K (VK) cycle in the (A) absence and (B) presence of warfarin. (A) The enzyme γ-glutamyl carboxylase (GGCX) (activity 1) with the cofactor vitamin K hydroquinone (VKH2) facilitates the transformation of peptide-bound glutamate (Glu) to γ-carboxyglutamate (Gla) residues and the subsequent synthesis and secretion of carboxylated VK-dependent proteins. The γ-carboxylation reaction results in the generation of VK epoxide (VK>0), which is reduced to VK quinone by the enzyme VK epoxide reductase (VKOR) (activity 2). VK quinone is then reduced to the VKH2 cofactor by 1 or more unidentified NAD(P)H-dependent reductases (activity 3), or possibly by VKOR itself (activity 2), to complete the cycle. (B) In the presence of a vitamin K antagonist (VKA) such as warfarin, VKOR (activity 2) is inhibited, resulting in the synthesis and secretion of inactive species of undercarboxylated proteins called proteins induced by vitamin K absence or antagonism (PIVKAs). Given sufficient input of vitamin K into the cycle, an alternative quinone reductase pathway (activity 3) can bypass the VKOR to provide the VKH2 substrate for GGCX and hence overcome the inhibitory action of warfarin, even under extreme blockade. Reproduced with permission from Shearer and Okano.3


REFERENCES
Ibrutinib and Aspergillus: a Btk-targeted risk

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In this issue of Blood, Bercusson and colleagues report a mechanism underlying susceptibility to Aspergillus fumigatus infections in patients treated with the Bruton tyrosine kinase (Btk) inhibitor ibrutinib. This drug has been paradigm-shifting in a number of B-cell malignancies, which has led to widespread use in the diseases for which Food and Drug Administration approval currently exists, and clinical trials of the drug alone and in combination in these and many other malignancies. However, although well tolerated in the majority of patients, a few troublesome toxicities have become apparent as use of the drug has expanded. One such toxicity is a relatively high rate of A fumigatus infections. Although patients with hematologic malignancies are certainly at risk for opportunistic infections, A fumigatus infections at a higher rate than expected have been noted in multiple prospective studies, most notably a rate of 39% in patients on a trial of ibrutinib in primary central nervous system (CNS) lymphoma who were concurrently treated with corticosteroids. Although the incidence in other hematologic malignancies is likely quite a bit lower (2.5% in a large institutional cohort of 566 patients treated over 1225 person-years), this finding has been worrisome. A previous study by this group, exploring the risk of A fumigatus in patients treated with calcineurin inhibitors, demonstrated that Btk is required for the macrophage inflammatory response that clears A fumigatus from the airways, and a separate study showed that Btk null mice infected with A fumigatus developed pneumonia, similar to what is seen in macrophage-deplete models. However, the implications for these findings in patients where Btk is present but pharmacologically inactivated were previously unknown.

In this study, the authors show that the macrophage activation induced by A fumigatus exposure is inhibited by ibrutinib, and similarly, ibrutinib inhibits nuclear translocation of NFAT and NF-kB in these cells, which has previously been shown to be critical to neutrophil recruitment (see figure). Furthermore, human monocyte-derived macrophages (hMMDMs) and alveolar macrophages are unable to produce tumor necrosis factor-a in response to A fumigatus after Btk knockdown, and galactomannan production is inhibited in ibrutinib-treated hMMDMs. However, macrophage phagocytosis is not affected by ibrutinib.

This study is significant because it both points to a mechanism behind an important clinical observation and extends the knowledge of the complex interplay between ibrutinib and the innate immune microenvironment. Data so far on the effects of ibrutinib on innate immune cells have been quite mixed. Ibrutinib has been shown to limit natural killer cell antibody–dependent cellular cytotoxicity in vitro; however, other data suggest that when cocultured with monocytes, chronic lymphocytic leukemia (CLL) killing is not affected by ibrutinib. As well, there are data showing that ibrutinib promotes the activity of the CLL-supporting nurselike cells, but patient data have also shown that ibrutinib disrupts the interaction between macrophages and CLL cells in the bone marrow microenvironment. Also, there are data to suggest that ibrutinib impairs macrophage phagocytosis, but other data that show that monocyte phagocytosis is not affected. Overall, these data serve to highlight the complicated nature of the interplay between Btk and the tumor microenvironment and show the importance of continued efforts in this area and in the construct of in vitro conditions that mimic the complete microenvironment.

Clinically, the finding that A fumigatus infections in patients on ibrutinib are due to a specific defect in immunity induced by this drug is quite significant. Although the absolute risk is certainly low, it will be important to identify those patients at particularly high risk for infection. It appears from the CNS lymphoma trial that patients taking concomitant corticosteroids are at especially high risk. Other risk factors that have been identified include high number of prior therapies, diabetes, and liver disease. Unfortunately, antifungal prophylaxis is not simple with ibrutinib, because most antifungal agents inhibit CYP3A4 and thus raise levels of ibrutinib. Further work will be necessary to definitively determine which patients are likely to derive benefit from antifungal prophylaxis, and potentially which patients would be better served with alternative therapies. Although this risk certainly does not dampen enthusiasm for this effective drug, it serves as a reminder that no therapy is without risk, and continued work to develop the optimal agents and strategies for treating our patients with hematologic malignancies must remain a top priority.

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