CLL Foundation
Final Progress Report
Screening for Inhibitors of Bcl-2-IP_3R Interaction
Clark W. Distelhorst
November 5, 2014

BACKGROUND AND GOALS

Funding from the CLL Foundation has enabled our work and produced success in our effort to develop a novel therapeutic agent for CLL. The work is based on our discovery that the Bcl-2 protein regulates intracellular calcium homeostasis and signaling in lymphocytes (1,2). Accordingly, there is now considerable evidence that cancer cells remodel intracellular calcium homeostasis and signaling to promote their own survival (3). We find that one of the principle mechanisms involves the interaction of Bcl-2 with IP_3Rs (inositol 1,4,5-trisphosphate receptors), intracellular calcium channels located on the endoplasmic reticulum (ER) (4, 5, 6). CLL cells, which uniformly express abnormally elevated levels of Bcl-2, take advantage of Bcl-2-IP_3R interaction to prevent IP_3R-mediated calcium signals that induce programmed cell death (apoptosis) (7). Thus, CLL cells live longer than normal lymphocytes, which express Bcl-2 but at much lower levels than CLL cells.

Having discovered this important Bcl-2 function, we undertook sustained work toward developing a novel therapeutic agent that inhibits Bcl-2-IP_3R interaction and thereby kills CLL cells. The first step was to identify the Bcl-2 binding site on the IP_3R and then to develop a synthetic decoy peptide that inhibits Bcl-2-IP_3R interaction (6, 8). This peptide, which we now call BIRD-2 (Bcl-2 IP_3R Disruptor-2) (formerly referred to as TAT-$IDP_{DD/AA}$), induces apoptosis in primary human CLL cells but not in normal lymphocytes (7). Moreover, in collaboration with other investigators we find that BIRD-2 also induces apoptosis in diffuse large B-cell lymphoma (9). This provided us with proof-of-principle evidence of Bcl-2-IP_3R interaction as a potential therapeutic target for treating CLL. Therefore, we undertook to identify lead compounds that function like BIRD-2 to disrupt Bcl-2-IP_3R interaction and kill CLL cells.

Experiments Performed and Findings

In the succeeding steps we have performed a high throughput screen of a drug-like compound library, followed by secondary and tertiary screens, with the goal of identifying lead compounds that will form the basis for medicinal chemistry and animal testing before proceeding to clinical trials in humans. The successive steps are summarized here:

- Step 1: A high-throughput screen of a 25,482 drug-like compound library was performed, identifying 148 compounds as hits at 6.5 μM in the primary single-point screen with activity >30% relative to apoptosis induced by 10 μM BIRD-2, screened in triplicate for hit confirmation. 320 additional compounds chosen by virtual screening were also screened in triplicate.
- *Step 2:* Triplicate hit confirmation and dose response assessment was performed, narrowing the number of hits from 148 to 39.
- *Step 3:* The remaining 39 compounds were tested in triplicate at 10 concentrations for induction of apoptosis in the B-cell malignancy line relative to apoptosis induced by 10 μM BIRD-2. This dose response screen was performed at two concentration ranges (0.041 – 20.5 μM and 0.008 – 3.95 μM) using 0.4% and 0.1% final DMSO concentrations. On this basis, the number of useful hits was narrowed to 28.
- *Step 4:* The 39 hits were screened for their effects on viability of primary human CLL cells. The approach eliminated hits that failed to induce apoptosis in CLL cells, leaving 28 compounds for further testing.
- *Step 5:* The remaining 28 hits were individually analyzed by single cell digital imaging to assess cytoplasmic calcium elevation. Of the 28 compounds tested, 6 induced calcium elevation to a degree comparable to the induction by 10 μM BIRD-2. These assays were repeated in Jurkat human leukemia lines, the Bcl-2 overexpressing murine lymphoma line WEHI7.2, and in freshly isolated primary human CLL cells. This narrowed the field of interesting compounds from 28 to 6.
- *Step 6:* The 6 remaining hits were individually analyzed to determine if their action mimics that of BIRD-2 based on demonstrating IP_3R dependence of the observed calcium response. This involved three separate assays performed in parallel: (a) determining the effect of the phospholipase c inhibitor U73122 on the calcium elevation induced by each compound; and, (b) comparing calcium responses to each compound in

wild type and IP_3R triple knockout DT40 chicken B-cell lymphoma cells; (c) determining whether or not each individual compound induces a decrease of calcium concentration in the endoplasmic reticulum of a Bcl-2 positive HEK-293 line expressing the FRET-based calcium indicator D1ER. These assays confirmed the 6 hits as having effects on intracellular calcium comparable to that observed in response to BIRD-2.

- *Step 7:* Surface plasmon resonance (SPR, or Biacore) assays were performed to assess the interaction of each of the preceding compounds with the BH4 domain of Bcl-2. The BH4 domain is the region of Bcl-2 that binds to IP3Rs and it is known from our earlier work that BIRD-2 binds directly to the BH4 domain. Thus, we wanted to determine which of the 6 compounds, like BIRD-2, bind to the BH4 domain. On initial pass 4 or the 6 compounds bound to the synthetic biotin-tagged BH4 domain bound on streptavidin-coated plates. The top hit bound to the BH4 domain with a K_D of 5.6 μM.

Significance of Findings

Steps 6 and 7 were mainly performed through the generous support of the CLL Foundation. Through this work we now have a lead compound that works in the single micromolar range, binding to the BH4 domain of Bcl-2 with a K_D of 5.6 μM. It disrupts intracellular calcium homeostasis and induces apoptosis in primary human CLL cells. It also displays synergistic activity when combined with the BH3-mimetic compound ABT-263, a compound that inhibits Bcl-2 by a completely different mechanism. The compound is a piperazine derivative that was developed by the pharmaceutical industry and even tested in animals in the first half of this decade. It appears the compound did not fulfill expectations for use in a non-malignant condition and was never tested as a potential anti-cancer agent. Numerous analogues were also produced at the same time which we anticipate to be of considerable value as we work to optimize this drug for treatment of CLL.

This is a breakthrough discovery of a compound that validates our central hypothesis that Bcl-2-IP3R interaction can be targeted as a novel therapeutic approach for CLL. It is a great starting point for drug discovery. This drug discovery process is critically important since CLL remains an incurable malignancy for the most part. New agents (*e.g.,* ibrutinib) have been met with considerable excitement, but now that we are beginning to use it at our own institution we find it is not without significant side effect and observed therapy resistance developing, often accompanied by very aggressive recurrence. Moreover, Abbott has invested heavily in development of their form of Bcl-2 inhibitor (*e.g.,* ABT-199, the current version). However, ABT-199 only inhibits one mechanism by which Bcl-2 inhibits apoptosis, leaving a wide-open gap in mechanistic targeting. Our compound will fill this gap, enabling us to inhibit both mechanisms by which Bcl-2 inhibits apoptosis, than thus predictably enhancing therapeutic efficacy of both agents in CLL.

Future Directions

We have been provided with a small remaining quantity of our lead compound, along with its chemical structure. We now intend to perform LC-MS on the remaining compound we have to verify structural identity, followed by collaboration with a medicinal chemist to prepare more of the compound and to prepare analogues for comparison with the lead compound in terms of binding affinity for the BH4 domain of Bcl-2 (SPR/Biacore experiments) and functional activity in primary human CLL cells and in an animal model of CLL. Following successful fulfillment of these goals we will move toward a clinical trial in patients with relapsed, refractory CLL.

References

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