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Supplemental Information

Probing the Probes: Fitness Factors

For Small Molecule Tools

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In this supplemental section we highlight briefly some chemical probes for a range of additional oncology targets, of different types, that are of current interest to both basic and drug discovery research.

The challenge of protein-protein interactions: Probes for p53 and BCL2

The p53 protein plays a key role as the guardian of the genome. Inactivation of the p53 tumor suppressor in human cancers is extremely widespread and the p53 pathway has therefore been a major area for biological studies. Increasingly, cell-based screening has been used to uncover new potential drug targets linked to p53 function, either using or generating new chemical probes in the process (Brown et al., 2009). These include compounds that inhibit the binding of the negative regulator MDM2 protein to p53, such as the dihydroimidazole nutlin-3 (Vassilev et al., 2004; for the chemical structures of nutlin-3 and other compounds in this section see Supplementary Figure 1). Nutlin-3 is of note as an early example of specific inhibition of a protein-protein interaction by a small molecule, now in Phase I clinical trials. While nutlin-3 mimics the binding of three critical 'hot-spot' residues of p53 to a groove on the surface of MDM2, the p53-MDM2 interaction can also be disrupted by compounds that bind to the surface of p53, e.g. the (bisthienyl)furan RITA (Issaeva et al., 2004). Both modes of inhibition lead to reactivation of wild-type p53.

The activity of specific p53 mutants can be rescued with small molecules such as the surprisingly undecorated quinuclidinone PRIMA-1, of which an analogue APR-246 is in Phase I clinical trials, that binds into a cleft formed by the Y220C missense mutation, stabilizing functionally active folding of the protein (Bykov et al., 2002). Although exhibiting some limitations of selectivity and stability, an early chemical probe identified by phenotypic screening, the iminobenzothiazole pifithrin-α, did much to stimulate the field (Komarov et al., 1999; Walton et al., 2005). The above agents and others have been widely used as probes in p53 research.

Another important protein-protein interaction that is important for cell survival, is highly relevant to cancer therapy and has succumbed to inhibition by a non-peptide is the interaction of BCL2 and BH3 domain proteins. This interaction is blocked by the BH3 mimetic ABT-737, now in clinical trial (Oltersdorf et al., 2005). In a careful study using appropriate cells to compare ABT-737 with other putative BCL2-targeting agents – obatoclax, gossypol, apogossypol, EM20-25, chelerythrine – only ABT-737 specifically targeted BCL2 in cells. Thus ABT-737 appears to be the best probe for inhibiting BLC2 in cells (Vogler et al., 2009). Interestingly this high molecular weight sulfonamide defies all but one of the Lipinski rules of five.

Chromatin modification

The control of gene expression through dynamic chemical modification of chromatin proteins by acetylation, methylation, phosphorylation, ubiquitination, sumoylation and poly(ADP-ribose)ylation is important in cell biology and offers many opportunities for therapeutic intervention to treat cancer (Zheng et al., 2008). Histone deactylases (HDACs) occur in both the nucleus and cytoplasm, with class I enzymes predominantly found in the nucleus and class II enzymes more widely spread and often modifying non-chromatin proteins such as tubulin and HSP90. The natural product trichostatin A was an early chemical probe for HDAC inhibition (Yoshida et al., 1990), and was subsequently shown to inhibit tubulin deacetylation as well (Blagosklonny et al., 2002). Cell-based screening of a diversity-oriented chemical library identified compounds such as tubacin, able to selectively inhibit tubulin deacetylation (Haggarty et al., 2003). Many HDAC clinical candidate inhibitors have since been developed, most making use of a hydroxamic acid warhead or other zinc-chelating group to interact with the metalloenzyme, and there are now two approved clinical agents, the hydroxamic acid vorinostat (SAHA) originating from a series of compounds discovered in a cell-based screen for inducers of the differentiation of transformed cells (Richon et al., 1996), and the depsipeptide romidepsin (Ueda et al., 1994).

A recent chemical biology study using a panel of hydroxamic acid chemical probes has shown that while HDAC isoform specificity is varied, there is a general lack of class II HDAC chemical probes (Bradner et al., 2010) Screening of a focussed chemical library identified the inhibitor pandacostat which, although containing structural elements that might confer a risk of non-specific activity (polyphenolic substitution and a potentially reactive benzylidinehydrazide), represents an early probe for broad-spectrum class I and class II HDAC inhibitor activity (Supplementary Figure 2).

Probes for other classes for chromatin modifying enzymes are also of current interest including sirtuins, histone acetyltransferases, histone methyltransferases and histone demethylases (Zheng et al., 2008; Cole et al., 2008).

Phosphatases, PARP and tankyrase

Phosphatases are known to be challenging targets but interesting probes are beginning to emerge. In a study of the oncogenic phosphatase PPM1D, early potential tool compounds were identified by screening; despite its Michael acceptor potential, the thienylidene cyclopentanone CCT007093 phenocopied RNAi silencing of PPM1D by selectively killing those cancer cells with amplified and overexpressed PPM1D in a P38-dependent manner, while also exhibiting further mechanism-based biomarker evidence of on-target effects (Rayter et al, 2008). CCT007093 exhibited selectivity for PPM1D compared to the most closely related PPM1A phosphatase. This is another example of exploiting oncogene addiction, in this case dependence on PPM1D, with inhibition of its function causing death specifically in those cancer cells that amplify, overexpress and are addicted to it .

The concept of 'synthetic lethality' has been known in genetics for some time (Hartwell et al., 1997). It is used when a combination of loss of function mutations in two genes leads to cell death whereas single mutations are viable. The first pharmacologic exploitation of synthetic lethality that has progressed to the clinic is the killing of cancer cells with mutations in BRCA1

and 2 genes, which are important for DNA double-strand break repair by homologous recombination, by inhibitors of the DNA repair enzyme poly[ADP]ribose polymerase (PARP) such as olaparib (Farmer et al. 2005; Fong et al., 2009). PARP probes with varying isoform selectivities are now of interest. Thiopyranopyrimidinone inhibitors of the related PARP enzymes tankyrase 1 and 2, such as XAV-939, were discovered using chemical genetic screening and proteomics with active and inactive analogs, and were found to block the oncogenic WNT pathway by stabilizing axin and destabilizing β-catenin (Huang et al., 2009b). This opens up a new area for probes and therapeutics acting on a hitherto difficult to inhibit cancer pathway.
Of note is the fact that is that, in addition to cases discussed in the main text, a number of the above agents represent examples of a context-dependent phenotypic outcome in response to probes or drugs. It is only with the right combination of inhibitor and genetic abnormality that selective cell death is seen.

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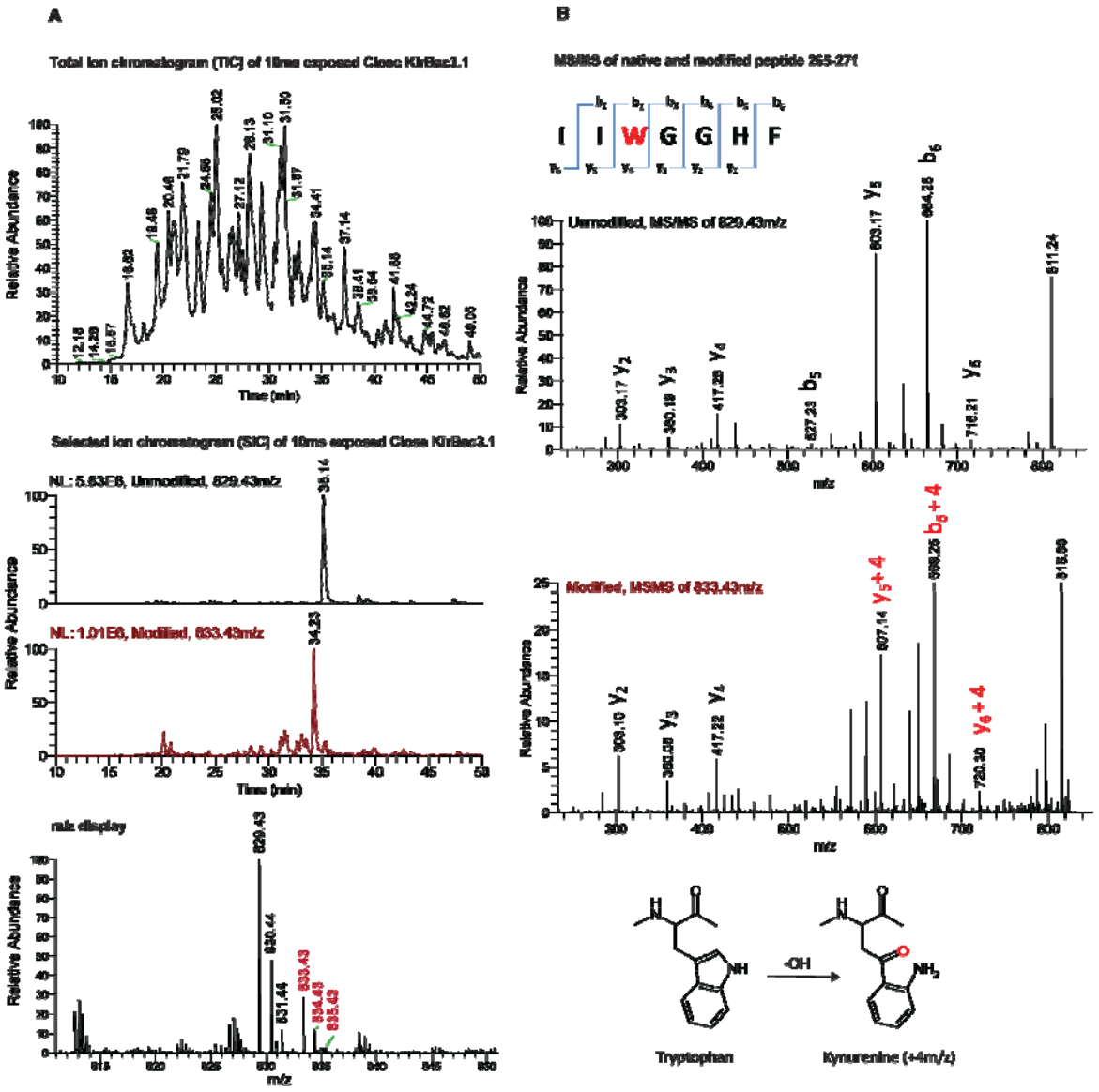

Fig. S1. Example of LC-LTQ-Orbitrap-MS data used to determine modification rate constant and identify site of modification. (A) The top panel shows a typical total ion current chromatogram of a peptic digest of X-ray irradiated (10ms) Closed KirBac3.1 represented by software XCalibur 2.01 (Thermo Electron) with scan filter for the range 400-1800m/z. The middle panel shows the selected ion chromatogram for the native (unmodified) and modified (+4 m/z shift, single peak) peptide 265-271 and their relative abundance after 10ms of X-ray irradiation. The bottom panel shows the m/z display of $(M+H)^+$ charge state and corresponding isotopic distributions. (B) MS/MS fragmentation of native and modified peptide 265-271, which shows +4 modification at W267 (kynurenine formation). The fraction of unmodified peptide at any exposure is calculated by the following equation:

$$Fraction\ Unmodified = \frac{Unmodified\ peak\ area}{Unmodified\ peak\ area + Modified\ peak\ area}$$

The peak areas are calculated from the selected ion chromatogram. A time evolution of SICs are used to calculated fraction unmodified vs. exposure time. Hydroxyl radical modification rate constants are determined from the single exponential curve fitting as described in Figure 1*B* and Methods.

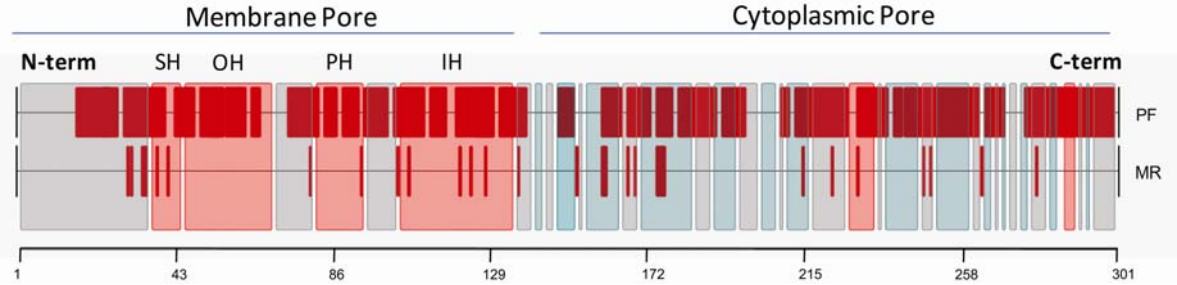

Fig. S2. Sequence Coverage: 92% sequence coverage was obtained from the LC-MS analysis of pepsin fragments (PF)(Figure S1 and Table 1). Primary sequence of KirBac3.1 covered by the PFs and location of modified residues (MR) identified by MSMS are marked in red blocks. The secondary structure consisting of loops, helix and sheets are represented by translucent grey, red and green background respectively. SH,OH,PH,IH represents Slide Helix, Outer Helix (TM1), Pore Helix and Inner Helix (TM2) respectively. Position of the modified residues in the three dimensional structure of KirBac3.1 are represented in Figure 1*C*.

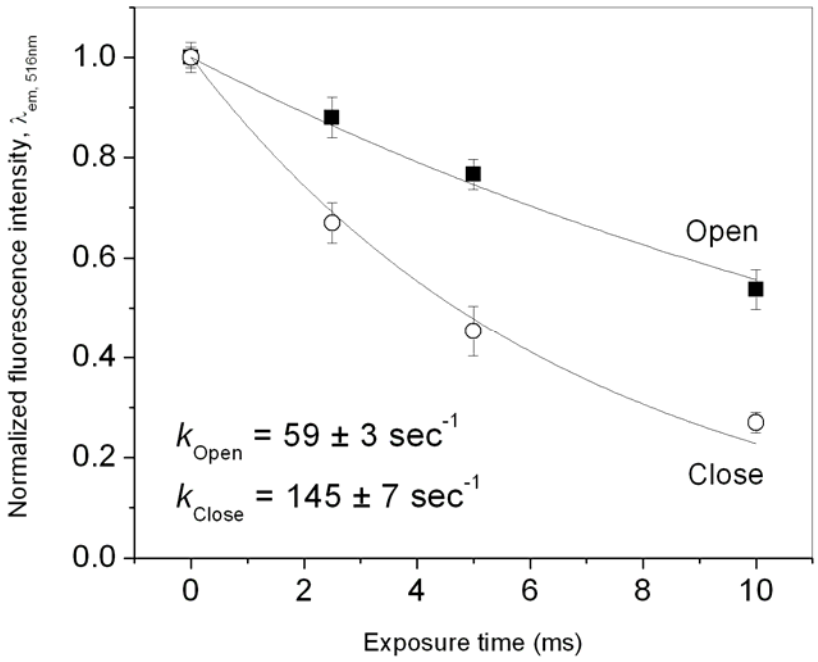

Fig. S3. Alexa 288 radiolysis and determination of X-ray dose: Dose response plots of 2µM Alexa in presence of 5µM closed and open KirBac3.1 solution in 50mM $MgCl_2$, 150mM KCl, 0.02% Tri-DM, 10mM Na-Cacodylate pH 7 and 1mM EDTA, 150mM KCl, 0.02% Tri-DM, 10mM Na-Cacodylate pH 7 respectively. Alexa solution in 5µl volume inside a PCR tube were irradiated with focused X-ray of energy 5-12keV (mirror angle=5.5, bender value = 8.0, ~ 10 fold more intense than the unfocused bending magnet source) for 0-10ms. After irradiation the solution is diluted (1:500) prior to fluorescence analysis. A Turner Biosystems TBS-380 fluorometer is used to determine the emission intensity at 516nm with an excitation wavelength of 496 nm. The solid line represents the fitting of data to a first-order reaction kinetics [$Y=A*\exp(-k.t) + y_0$, A=1 and y_0=0]. The rate constant k, is a measure of effective hydroxyl radical dose on the sample. The EDTA in the open state sample is a •OH radical scavenger and reduces the X-ray dose by a factor of ~ 2.45. The rate constant k_{open} is corrected by multiplying the factor 2.45 ($k_{open\text{-}corrected}$ = k_{open} X 2.45) and ratio of $k_{open\text{-}corrected}$ and k_{closed} was determined (Figure 1B and Table 1).