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High throughput discovery of heteroaromatic-modifying enzymes allows enhancement of novobiocin selectivity†

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Glycosylated analogues of novobiocin, discovered using a broad library of enzymes, have 100-fold improved activity against breast, brain, pancreatic, lung and ovarian cancers and ablated associated off-target activity leading to an up to 2.7 × 10^4 fold increase in selectivity.

Novobiocin is a member of the aminocoumarin family that acts as an inhibitor by binding to the GyrB subunit of DNA gyrase (type II topoisomerase)[1] and also possesses weak anti-cancer activity by binding to the Hsp90 chaperone protein.[2] The clinical use of this class of compound has been restricted due to their low water solubility[3] and toxicity *in vivo*.[4] Modifications of aminocoumarins have allowed some improvements in activity,[5] however, to date such modifications have required redesign of the novobiocin scaffold from scratch and cannot be performed at a late synthetic stage.

Glycosyltransferases (GTs) catalyse the transfer of sugar moieties from active donor molecules to specific acceptor molecules forming glycosidic bonds. GTs are important tools in the synthesis of possible drugs.[6] The exact identity and pattern of glycosyl moieties can influence pharmacology/pharmacokinetics, invoke biological specificity at the molecular/tissue/organism level and even define the precise mechanism of action.[7] In nature, the attachment of sugars to small molecules is used to mediate targeting, or modify the mechanism of action and/or pharmacology.[8] The complexity of natural products often makes traditional chemical glycosylation strategies impractical for such ''glycodiversification''. The use of enzymes such as GTs can avoid the need for protecting groups and allow glycosides to be formed from a late stage intermediate with often very good efficiency and selectivity. Previous studies have successfully demonstrated the power of such glycorandomization/glycodiversification either through the use of wild-type enzymes[8,9] or structure-based enzyme engineering.[10] Strategies for the rapid discovery of synthetically useful GT activities against a given target are therefore of high value. Based on a previous MS-based High Throughput Screening (HTS) method,[11] we have now developed and describe here a nano-LC ion trap (NLCIT) MS method. The novel application of MS^n detection can be used to rapidly screen not only for formation efficiency but also the product structure.

As part of a programme to generate novel anticancer compounds we have explored strategies to enhance or rescue the activity of representative compounds such as the model target novobiocin. GTs are known to display activities that modify planar, cyclic and hydrophobic molecules similar to these targets.[9,12] We reacted 107 *Arabidopsis thaliana* GTs and 18 Oat GTs using α-UDP-glucose as donor against 81 acceptors (Fig. 1, SI Fig. 1†).

A total of 81 × 125 > 10 000 screening events with 125 plant GTs allowed the identification of a number of compounds that processed modified aromatics and heteroaromatics (See SI) such as flavonoids, coumarins, benzoic acids, cinnamic acid and zeatin analogues. Following detailed analysis, we were delighted to find eleven GTs that glycosylated novobiocin (SI Fig. 2†): 78D2 (4%), 71B8 (4%), 71B1 (15%), 88A1 (1%), 73C5 (62%), 73C6 (5%), 73C1 (8%), 73B3 (4%), 73B4 (1%), 76E1 (4%) from *A. thaliana* and 20n10 (3%) from oat. NLCIT (Fig. 1) simultaneously revealed exquisite regioselectivity from glucosylated-novobiocin for modification of the 4′-OH of the aminocoumarin ring. Glycosylation of the precursor of novobiocin has been studied extensively.[13] However, to date, no glycosylation on novobiocin has been reported. After isolation from synthetic scale reactions (SI Scheme 1†), all structures were unambiguously confirmed.

Many reports have shown that GTs can have broad promiscuity with respect to both donor and acceptor.[9,10] It was also found through the screen that identified GTs could use a range of donors (up to 9, data not shown). However, only α-UDP-glucose could be used by GTs when novobiocin acted as an acceptor; other donors like α-UDP-galactose,

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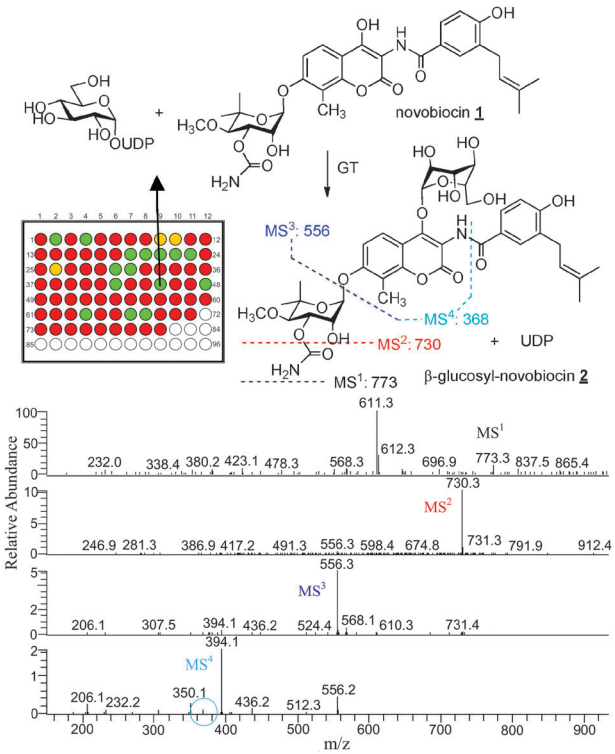

Fig. 1 NLCIT HTS method, glycosylation of novobiocin and structure elucidation. Top: HTS screen plate results of 73C1 indicated in colour: Green, positive; Red, negative and Amber, intermediate. Green spot at position 45 (novobiocin) indicates that novobiocin can be glycosylated by 73C1. Scheme shows the glycosylation reaction and possible MS fragments (MS^1–MS^4); Bottom: Structure elucidation of *β*-glucosyl-novobiocin (**2**) using ion trap MS. Peak (773) indicates the formation of **2**. The glycosylation position can be determined by MS^n. The MS^4 (773->730->556->368, 394) at 368 (cycle) delineates regioselectivity and indicated that the D-glucose residue was added onto the 4′-OH of the aminocoumarin ring.

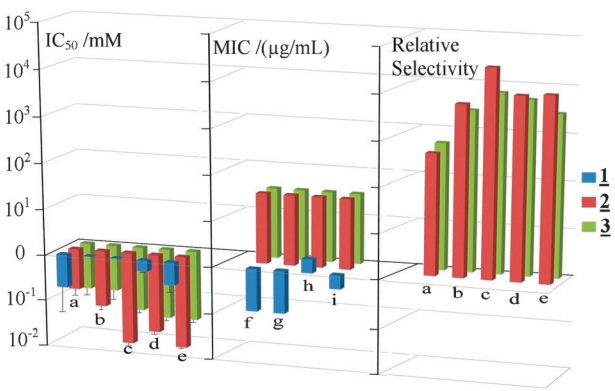

Fig. 2 Activity and selectivity of glycosylated novobiocins compared with novobiocin. Left: anticancer activity (IC_{50}), (a)–(e) represent ovarian, lung, breast, brain and pancreatic cancer, respectively. Centre: MIC result. (f), *S. aureus* NCTC 12981; (g), *S. aureus* NCTC 13373; (h), *E. faecalis* NCTC 12697 and (i), *E. faecalis* NCTC 13379 as proxy of topoisomerase. Each MIC was determined independently on three occasions and, where an endpoint was determined, varied no more than two-fold between values. Right: Relative selectivity of the anticancer activity of **2** and **3** compared to **1** (MIC with *S. aureus* as a reference).

α-UDP-GlcNAc, α-GDP-mannose, and *β*-GDP-fucose did not show activity.

Unmodified novobiocin binds to the Hsp90 C-terminal domain weakly and has poor activity.[2] Although they are not yet in a position to compete with their N-terminal binding counterparts, *e.g.* 17-AAG, novobiocin based C-terminal inhibitors have shown superior activities against various cancer cell lines. For example, F4 demonstrated superior activity to 17-AAG in inducing apoptosis in prostate cancer cell lines;[5a] KU135 inhibits Jurkat T lymphocyte cell proliferation and promotes apoptosis more potently than 17-AAG.[14] Using the products of the synthetic enzyme screen, we found that a single glycosylation at the 4′-hydroxy moiety of the coumarin ring increased significantly the anti-proliferative activity against human ovarian, lung, breast, brain and pancreatic cancer cell lines (Fig. 2, SI Table 1†). Glucosylation of novobiocin (**2**) increased the activity against breast cancer by 100-fold, lung cancer by 17-fold, brain cancer by 29-fold and pancreatic cancer by 32-fold respectively. Prompted by this exciting result, a variant of **2**, *β*-galactosyl novobiocin (**3**) was prepared with a subtle configurational change at just one stereogenic centre 4‴-OH (SI Scheme 1†). The modification in **3** increased those activities to a lesser extent by 25-fold, 10-fold, 19-fold and 11-fold, respectively, suggesting an important role for glycosylation in both enhancing and modulating activity (up to 4-fold). Interestingly, both compounds only showed a limited increase of activity against the A2780 ovarian cancer, with increases of only 1.4-fold and 1.9-fold. The exact mechanisms of how an individual sugar leads to these changes are yet to be understood; early protein modeling suggested enhanced binding mediated by sugar residues to the Hsp90 C-terminal domain peptide residues (SI Fig. 7 and 8†). The *in vitro* activities of novobiocin (**1**), and glycosyl novobiocins (**2** & **3**) were evaluated against several bacterial strains that are sensitive to topoisomerase inhibition (SI Table 2†). Glycosylation reduced this activity up to 266-fold. Glycosylation therefore separates the anticancer activity and antibacterial (as a proxy for topoisomerase) activities up to 2.7×10^4 fold (Fig. 2) as compared to novobiocin.

In conclusion, the NLCIT HTS method disclosed here rapidly elucidated substrate specificity and product structure *via* MS^n fragments. Eleven catalysts were discovered that allowed modification and "reclamation" of novobiocin as a model target. Previously, poor affinity to Hsp90 and higher affinity for type II topoisomerases has prevented novobiocin from being evaluated as a clinically useful Hsp90 inhibitor.[15] A 2.7×10^4 fold separation of wanted activities from unwanted was achieved efficiently and rapidly, suggesting a useful alternative strategy for anticancer drug discovery based on the use of sugars as prosthetics.

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