Quantitative electrospray mass spectrometry for the rapid assay of enzyme inhibitors

Jiangyue Wu, Shuichi Takayama, Chi-Huey Wong and Gary Siuzdak

Background: Combinatorial chemistry has become an important method for identifying effective ligand–receptor binding, new catalysts and enzyme inhibitors. In order to distinguish the most active component of a library or to obtain structure–activity relationships of compounds in a library, an efficient quantitative assay is crucial. Electrospray mass spectrometry has become an indispensable tool for qualitatively screening combinatorial libraries and its use for quantitative analysis has recently been demonstrated.

Results: This paper describes the use of quantitative electrospray mass spectrometry for screening libraries of inhibitors of enzymatic reactions, specifically the enzymatic glycosylation by β-1,4-galactosyltransferase, which catalyzes the transfer of galactose from uridine-5′-diphosphogalactose to the 4-position of *N*-acetylglucosamine βOBn (Bn: benzene) to form *N*-acetyllactosamine βOBn. Our mass spectrometric screening approach showed that both nucleoside diphosphates and triphosphates inhibited galactosyltransferase while none of the nucleoside monophosphates, including uridine-5′-monophosphate, showed any inhibition. Additional libraries were generated in which the concentrations of the inhibitors were varied and, using mass spectrometry, uridine-5′-diphosphate-2-deoxy-2-fluorogalactose was identified as the best inhibitor.

Conclusions: This report introduces quantitative electrospray mass spectrometry as a rapid, sensitive and accurate quantitative assaying tool for inhibitor libraries that does not require a chromophore or radiolabeling. A viable alternative to existing analytical techniques is thus provided. The new technique will greatly facilitate the discovery of novel inhibitors against galactosyltransferase, an enzyme for which there are few potent inhibitors.

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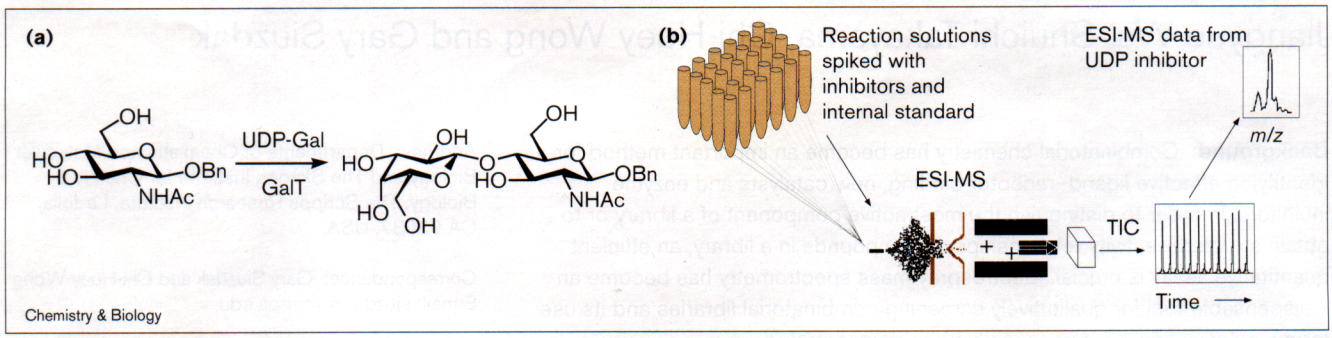
Introduction

Combinatorial chemistry has rapidly become a useful tool in a variety of chemical arenas and has been used to search for effective ligand–receptor binding, new catalysts and enzyme inhibitors [1–10]. In order to identify the most active component of a library or to obtain structure–activity relationships of compounds in a library, it is crucial that there is an efficient quantitative assay. But although there are several rapid qualitative assays [1,11,12], the quantitative assay methods, such as radiolabeling, nuclear magnetic resonance spectroscopy, fluorimetry, ultra-violet spectroscopy, affinity capillary electrophoresis and surface plasmon resonance [13,14], have, in certain cases, limitations and problems when used in rapid screening because they require extensive sample preparation and optimization time, high cost, biohazardous disposal, or large reaction volumes (ml) of sample.

Mass spectrometry has become an indispensable analytical tool for the characterization and qualitative screening of combinatorial libraries [15–18], and for selection assays [19,20]. Recently, we were successful in using matrix-assisted laser desorption/ionization mass spectrometry as an automated combinatorial approach for the optimization of extraction solvent systems [21]. In this paper, we report a similar approach for screening inhibitors of enzymatic reactions, specifically of enzymatic glycosylation. The approach described herein demonstrates electrospray mass spectrometry as a rapid and sensitive quantitative screening tool that does not require a chromophore or radiolabeling and thus provides a viable alternative to existing analytical techniques. Electrospray also offers many advantages over other mass spectrometric methods including its ability to analyze low-mass compounds, excellent quantitation and reproducibility, high sensitivity, simple sample preparation, automation, soft ionization, and the absence of matrix requirements [22–25]. In addition, because only product formation is monitored with respect to an internal standard, the quantitative screening procedure is straightforward, rapid, and independent of the inhibitor's ionization. To our knowledge, this is the first example of using electrospray mass spectrometry to rapidly and quantitatively screen

Figure 1



The galactosyltransferase reaction. **(a)** β-1,4-Galactosyltransferase (GalT) catalyzes the transfer of galactose from uridine-5′-diphosphogalactose (UDP-Gal) to the 4-position of *N*-acetylglucosamine βOBn (left; Bn, benzene) to form *N*-acetyllactosamine βOBn (LacNAcβOBn; right). **(b)** The galactosyltransferase reaction is monitored during the electrospray ionization mass spectrometry (ESI-MS) autosampling screening experiment. Each solution contains the enzyme, the reactants, an inhibitor, and an internal standard. Product formation (LacNAcβOBn) is monitored with respect to the internal standard (LacNAcβSPh; Ph, phenyl). TIC, total ion current; *m/z*, mass/charge.

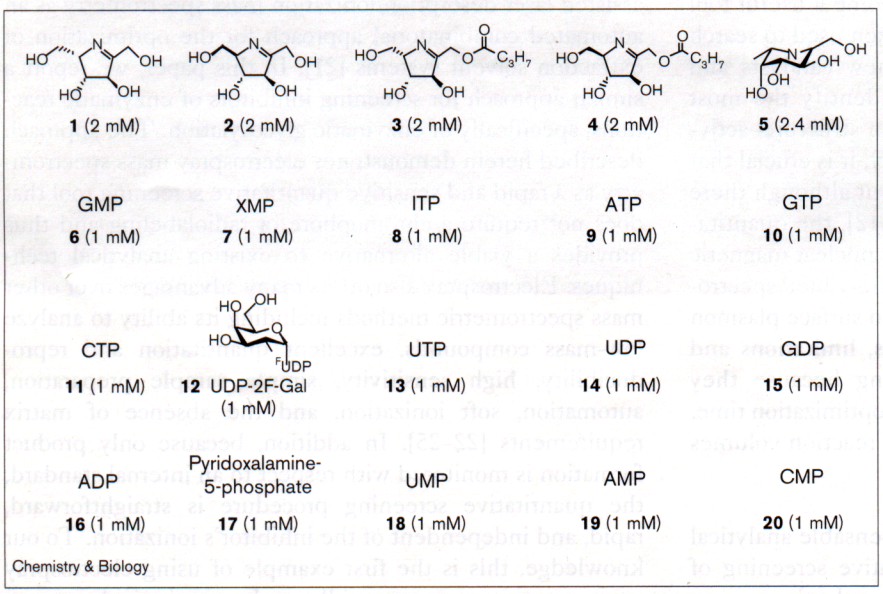
inhibitor libraries and to determine the inhibition potency of compounds.

Results and discussion

Cell surface carbohydrates are often associated with many specific recognition and signaling processes that are involved in important biological functions and diseases [26]. Inhibition of the enzymes involved in carbohydrate biosynthesis, such as glycosyltransferases, is, therefore, scientifically interesting as well as medically relevant. To date, however, only limited success has been achieved in the rational design of inhibitors of these enzymes [27–36], perhaps due to the lack of efficient and convenient analytical methods and the problem associated with the weak binding of sugars to their receptors. The application of combinatorial chemistry to generate libraries of potential inhibitors would allow many variations on a particular structural theme to be explored, and thus increase the likelihood of discovering new inhibitors of glycosyltransferases. For the quantitation of glycosyltransferase activity the current techniques, such as radioactive assays and spectrophotometrics assays, require separating compounds, the use of radiolabeled sugars, large-scale reactions, or the use of additional enzymes to indirectly measure product formation [35,37]. In addition, these techniques are not suitable for rapidly screening large libraries.

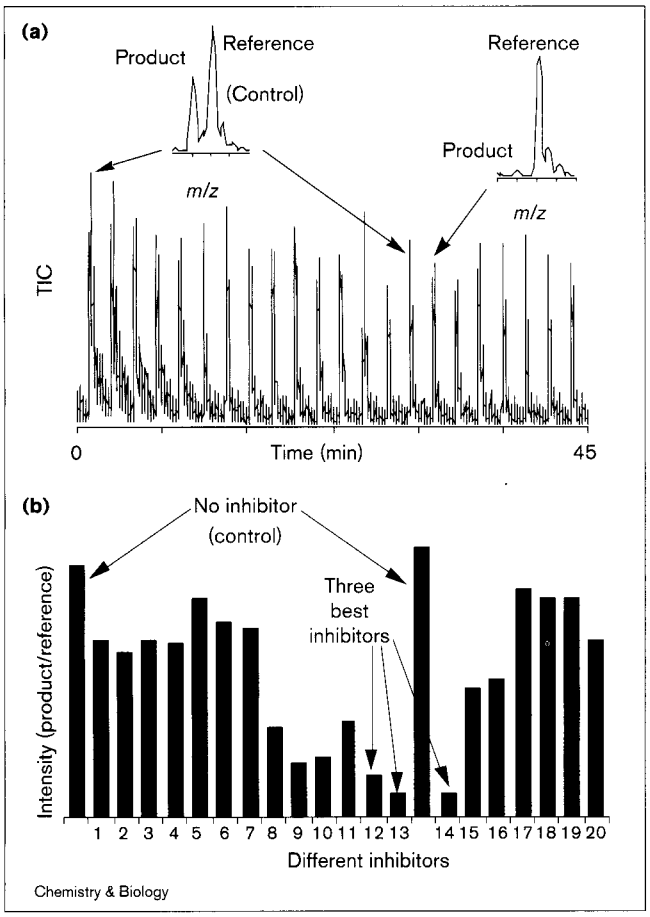
In this study, the inhibitors were screened against the enzyme β-1,4-galactosyltransferase, which catalyzes the transfer of galactose from uridine-5′-diphosphogalactose

Figure 2



The 20 inhibitor candidates in the first library and the concentration of each inhibitor used in the initial screening.

Figure 3

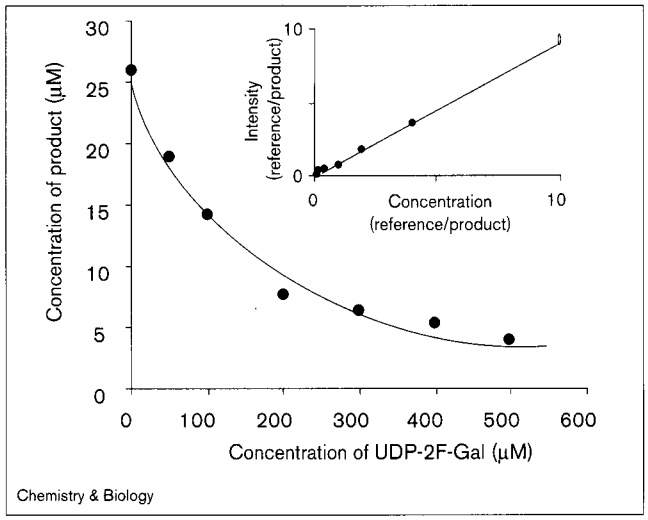


Mass spectrometry screening results. **(a)** The electrospray ionization TIC observed for the 22 reactions (each having a different inhibitor). Inserts are the mass spectra for the reactions with no inhibitor (left) and an inhibitor found to be effective (right). **(b)** A bargraph of the ratio of the intensities of the product and reference ions, generated from the mass spectrum for each of the potential inhibitors shown in Figure 2. The two control samples that contained no inhibitor are indicated.

(UDP-Gal) to the 4-position of *N*-acetylglucosamine βOBn (GlcNAcβOBn) to form *N*-acetyllactosamine βOBn (LacNAcβOBn) (Figure 1). LacNAc is a major component of glycoconjugates, which function as differentiation antigens, tumor-associated antigens and cell-recognition molecules that mediate inflammation and tumor development [38–40].

The initial inhibitor library contained 20 individual candidates (Figure 2; [41]). These 20 candidates and two control reactions were assayed individually in 22 parallel reactions (see Materials and methods section for details). The mass spectrometry screening results showed that both nucleoside diphosphates and triphosphates exhibit substantial inhibition in which three inhibitors, uridine-5′-diphosphate (UDP), uridine-5′-diphospho-(2-deoxy-2-fluoro)galactose (UDP-2F-Gal) [34] and uridine-5′-triphosphate (UTP) were the most potent (Figure 3).

Figure 4



The product of the galactosyltransferase-catalyzed enzyme reaction (Figure 1a) was monitored as a function of inhibitor concentration. The inset shows the correlation between the reference/product ratios of concentrations and ion intensities.

Although the inhibition caused by UDP and UTP [37] is well known, it is interesting to see from our mass spectrometric screening that other nucleoside diphosphates and triphosphates inhibit galactosyltransferase and that none of the nucleoside monophosphates, including UMP, showed any inhibition. The diphosphate moiety is very important for binding to the enzyme. It is also useful to note that the substrate was selected to remove inhibitor or substrate overlap of the mass/charge (*m/z*) for the product ions. To obtain a better comparison of the three most potent inhibitors and to demonstrate the applicability of this screening method to the measurement of inhibition potency, a second library was generated to determine the IC_{50} (50% inhibitory concentration) of UTP, UDP and UDP-2F-Gal, in which the concentrations of the inhibitors were varied (0, 50, 100, 200, 300, 400, 500 μM and extended to 1000 μM for UTP). The reaction conditions were the same as those described for the initial screening except that the reaction time was 10 min and the reaction was initiated by the addition of enzyme (see the Materials and methods section for details). The values are consistent with those based on radiolabeled assays: K_i = 460 μM [37] and 149 μM for UDP and UDP-2F-Gal [34]. The results showed the IC_{50} (within 90% confidence) to be 628 ± 8 μM for UTP, 260 ± 15 μM for UDP and 119 ± 13 μM for UDP-2F-Gal. UDP-2F-Gal was thus identified as the best inhibitor (Figure 4).

To explore the possibility of screening inhibitors even more efficiently, five potential inhibitors were pooled and screened simultaneously. The reaction mixture that

contained five inactive compounds (nucleoside monophosphates) showed no inhibition, whereas the reaction mixture containing four inactive compounds and one active inhibitor (UDP) showed potent inhibition. The compounds in the inhibited reaction mixture could then be individually analyzed to identify the actual inhibitor compound. Such strategies are able to increase the screening capacity of the already very rapid screening method several fold (from 1 inhibitor/2 min, 720 inhibitors/day to 5 inhibitors/2 min, 3600 inhibitors/day).

Significance

This report demonstrates that electrospray mass spectrometry is a practical approach for rapidly and quantitatively screening libraries of enzyme inhibitors and that it is sensitive and accurate enough to perform IC_{50} (50% inhibitory concentration) studies. The application of this new method for the rapid, direct, quantitative assay of galactosyltransferase makes this report especially noteworthy as conventional analytical methods for assaying this enzyme are time consuming, require separation of compounds, use radiolabeled sugars, give only indirect measurements, or require large reaction volumes. In short, the problems of existing assay methods have hindered the development of potent inhibitors against galactosyltransferase. Electrospray is superior to other mass spectrometry methods in terms of analytical specificity and accuracy because the sample preparation is simple and the technique provides direct measurements of product formation. Also, as the sensitivity of electrospray mass spectrometry has been observed at the femtomole level, it is likely that considerable improvements can be made to increase its sensitivity for small quantities of inhibitors. Overall, the method is straightforward and applicable to various enzyme–inhibitor systems and will aid the discovery of novel enzyme inhibitors.

Materials and methods

Initial screening

Reactions were performed on a 50 µl scale in 0.5 ml Eppendorf tubes. Each reaction mixture contained GlcNAcβOBn (1 mM; [42,43]), $MnCl_2$ (1 mM), β-1,4-galactosyltransferase (0.6 mU), UDP-Gal (0.4 mM) and the inhibitor candidates. The reaction was initiated by the addition of UDP-Gal and then allowed to stand for 20 min at 25°C. The reaction was quenched by addition of MeOH (0.4 ml) and finally, the reference compound LacNAcβSPh (4 µl of a 1 mM aqueous solution; [42,43]) was added. This mixture was used directly in the electrospray analysis.

Determination of IC_{50}

Reactions were performed on a 50 µl scale in 0.5 ml Eppendorf tubes. Each reaction mixture contained GlcNAcβOBn (1 mM), $MnCl_2$ (1 mM), β1,4-galactosyltransferase (0.6 mU), UDP-Gal (0.4 mM) and inhibitor (0, 50, 100, 200, 300, 400, 500 µM). The reaction was initiated by the addition of β-1,4-galactosyltransferase and was allowed to stand for 10 min at 25°C. The reaction was quenched by addition of MeOH (0.4 ml) and finally, the reference compound LacNAcβSPh (4 µl of a 1 mM aqueous solution) was added. This mixture was used directly in the electrospray analysis.

Automated electrospray mass spectrometry

The crude reaction products with internal standard were loaded into the high-performance liquid chromatography (HPLC) (Hewlett Packard, HP1090, Wilmington, Delaware, USA) autosampler and then delivered to the electrospray (Perkin Elmer SCIEX API100 single quadrupole, Foster Cite, CA, USA) mass spectrometer using methanol as the mobile phase. In all of the experiments the autosampler injector was programmed to deliver 10 µl of the sample volume with an injection draw rate of 83 µl/min. The flow rate of the mobile phase was set at 0.2 ml/min and the sample injection rate was set at one sample every 2 min. The sample solution from the HPLC was split at a ratio of 1:5 and introduced into the ionization source through a capillary at a flow rate 40 µl/min. The nebulizer gas was optimized and set at a rate of 1.6 l/min. An electrospray potential of 4200 V was applied to the interface sprayer to charge the sample droplets. A curtain gas of ultrapure nitrogen was pumped into the interface at the rate of 1.2 l/min to aid desolvation of the charged droplets and to prevent particulate matter from entering the analyzer. The orifice was set at a relatively low potential (50 V) to minimize ion fragmentation. The data were acquired at a step size of 0.2 *m/z* and 1.0 ms dwell time, and the analyzer was set to scan the mass range from 490 to 510 *m/z* to analyze for the sodium adduct of the product, *m/z* 496, and the reference, *m/z* 498.

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