

Discovery and Characterization of Protein-Modifying Natural Products by MALDI Mass Spectrometry Reveal Potent SIRT1 and p300 Inhibitors**

Susanne Holzhauser, Anja Freiwald, Christoph Weise, Gerd Multhaup, Chung-Ting Han, and Sascha Sauer*

Natural products are a rich resource for the development of chemical probes, functional foods (nutraceuticals), and pharmaceuticals.^[1] The remarkable structural diversity of natural product or biology-oriented compound libraries in particular argues for their use in applications^[1b,2] such as drug screenings.^[3]

Enzymes that can posttranslationally modify proteins or nucleic acids are attractive drug targets and include kinases and phosphatases,^[4] methyltransferases^[5] and acetyltransferases, and deacetylases.^[6] Recently, histone-modifying enzymes like the deacetylase sirtuin 1 (SIRT1) were suggested as drug targets for treating a variety of age-related disorders such as neuropathogenic diseases, metabolic diseases, and cancer.^[7] Compound screenings for SIRT1 modulators have revealed promising enzymatic activators such as the natural product resveratrol and the synthetic compound SRT1720. However, these findings are still highly controversial due to reported assay artifacts. The employed screening assays were based on fluorescence-labeled peptide substrates and resulted in the purported but artificial enzymatic activation of SIRT1. These findings have misled many researchers over the years.^[8]

Besides the generation of artifacts as observed in SIRT1 assays, there is a second general drawback of fluorescence-based assays which is broadly underestimated: Autofluorescence of compound libraries and in particular natural product libraries interferes with widely applied optical analyses^[9] such as time-resolved fluorescence resonance energy transfer (TR-FRET).^[10]

By contrast, mass spectrometry (MS) represents an attractive alternative as substrates are detected directly. Peptides are ionized and detected according to their mass to charge ratios (m/z).^[11] For example, deacetylation of a substrate peptide can be directly detected as a 42 Da mass peak shift (Figure 1).

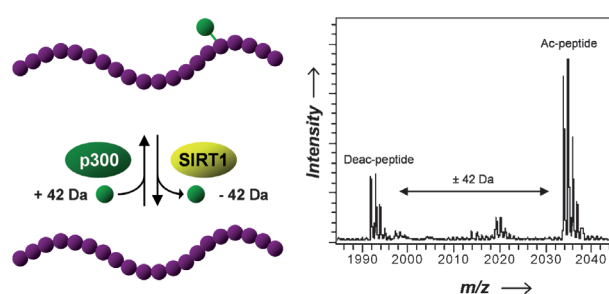


Figure 1. Deacetylation and acetylation of peptide substrates by SIRT1 and p300, respectively. Both deacetylation and acetylation result in a change in peptide mass of 42 Da, which can be observed in the mass spectra.

Electrospray ionization (ESI)^[12] and matrix-assisted laser desorption/ionization (MALDI)^[13] MS have become widely used techniques in basic biological research. ESI MS depends on the injection of dissolved analytes, and washing steps are required between sample injections to avoid cross-contamination. This procedure can limit throughput in practice. MALDI MS is based on the simple preparation of spatially distinct spots on a metal plate, each spot containing analyte and matrix molecules. Since many spots can be placed side by side on one plate, the consecutive ionization of analyte spots by a laser facilitates high-throughput detection with a reduced risk of cross-contamination. Although quantification was initially a challenging aspect in the context of MALDI MS, many quantification approaches have been developed since then, in particular for low complex samples.^[14] In recent years this has resulted in several novel quantification-based techniques, for example in genetic diagnostics.^[15] Moreover, the easy maintenance and operation of MALDI mass spectrometers makes this method attractive to non-experts in mass spectrometry.

Based on MALDI MS we have developed and validated a method for the high-throughput discovery of lead compounds and the characterization of compounds that can modify the deacetylase activity of SIRT1 (see the Supporting

[*] S. Holzhauser, A. Freiwald, Dr. C.-T. Han, Dr. S. Sauer
Otto Warburg Laboratory
Max Planck Institute for Molecular Genetics
Ihnestrasse 63–73, 14195 Berlin (Germany)
E-mail: sauer@molgen.mpg.de

S. Holzhauser, Dr. C. Weise, Prof. Dr. G. Multhaup
Fachbereich Biologie, Chemie, Pharmazie/Institut für Chemie und
Biochemie, Freie Universität Berlin
Takustrasse 3/Thielallee 63, 14195 Berlin (Germany)

[**] We thank B. Łukaszewska-McGreal and Dr. C. Weidner for support. This work is part of the PhD thesis of S.H. This work was funded by the German Ministry for Education and Research (BMBF, grant number 0315082), the National Genome Research Net (NGFN, grant number 01 GS 0828), the European Union ([FP7/2007-2011], under grant agreement no. 262055 (ESGI)), and the Max Planck Society.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201207325>.

Information, Figure S1). Furthermore we have adopted the assay principle for the reverse reaction, that is, acetylation by the acetyltransferase p300.

We tested the activity of the aforementioned purported SIRT1 activators resveratrol and SRT1720 by MALDI MS using various peptide substrates, either with or without fluorescence label. Consistent with recent studies using radioactive labeling^[8a,b] or HPLC,^[8c] we observed that different fluorescence labels produced diverging activation artifacts while the unlabeled natural peptide showed no SIRT1 activation but even inhibition (Figure 2).

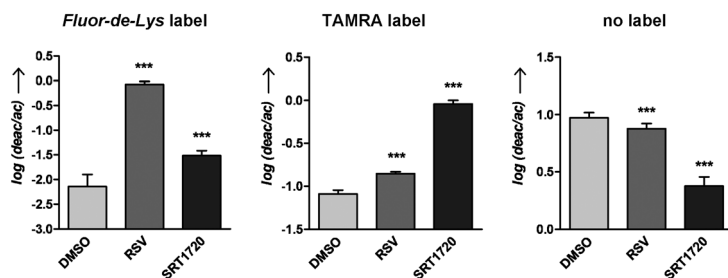


Figure 2. Comparison of SIRT1 activators by using different substrate peptides and MALDI detection. Substrates were fluorescently labeled and unlabeled peptides. Reactions contained 1% DMSO, treatments 100 μM resveratrol (RSV) and 100 μM SRT1720, respectively.

To optimize the procedure for screening we applied the unlabeled histone H4 peptide as a well-known target of SIRT1^[16] in a concentration of 0.5 μM . This is well below the K_m value ($(16 \pm 2) \mu\text{M}$; Figure S2) and makes it possible to detect competitive inhibitors.^[17] The degree of deacetylation was set at about 40% (10 nM SIRT1, 30 min; Figure S3 a) to enable robust detection of inhibitor activity. The quality of the assay was confirmed by a Z' -value^[18] of 0.5 (Figure S4). Moreover, we determined an IC_{50} value of $(0.26 \pm 0.02) \mu\text{M}$ for suramin, a known SIRT1 inhibitor, which is consistent with the literature value of $(0.297 \pm 0.01) \mu\text{M}$,^[19] and an IC_{50} value of $(0.58 \pm 0.03) \mu\text{M}$ for EX-527, another described SIRT1 inhibitor, which is also in the range of recently reported data.^[20] The accuracy of these values generated by MALDI was additionally validated by ESI MS (Table S1).

To calculate enzyme kinetics and compound potencies, exact quantification techniques are essential. Quantification with MALDI MS has been used successfully for complex peptide samples and other biological samples.^[21] We achieved the reliable quantification of acetylated and deacetylated peptides by generating peptide “calibration curves” (CC) as described in the Supporting Information, in which the measured values are plotted versus the calculated values (Figure 3 and Figure S5), and this was used to correct the experimental data. For uniform sample detection we optimized preparation conditions by applying a homogeneous ultrathin layer (UTL).^[22] A more detailed description and a discussion of peptide quantification are provided in detail in the Supporting Information.

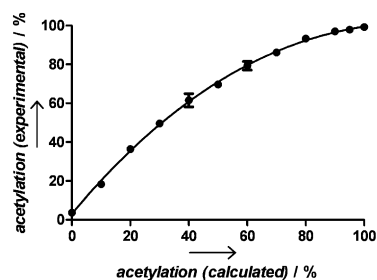


Figure 3. Peptide “calibration curve” depicts the relationship of the measured peptide ratios to the calculated values and can be used to interpolate measured sample values. 12 MALDI measurements were conducted per data point.

Using the described assay principle we screened a library of 5500 natural products^[3a] for SIRT1 modulators. To increase the throughput we pooled five compounds in each well and used 384-format MALDI target plates. Pools containing potential hits were retested as single compounds. While the reaction and sample purification took about half an hour each, in a single automated MS run of about two hours we could detect the activity of more than 1000 compounds.

Sample throughput can be further enhanced by optimizing the compound pooling size using the following group testing equation [Eq. (1)].^[23]

$$f(n) = (1-p)^n - (1/n) \quad (1)$$

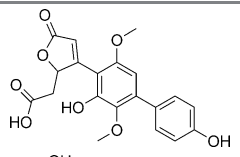
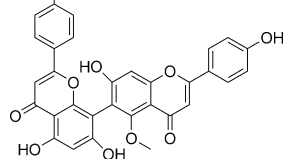
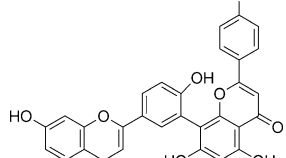
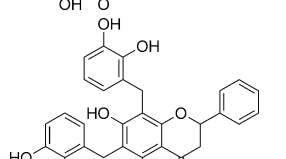
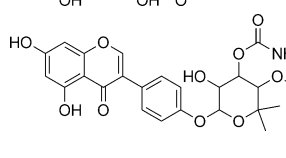
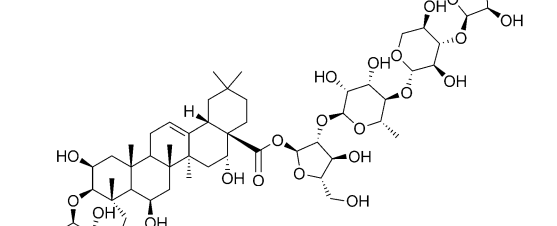
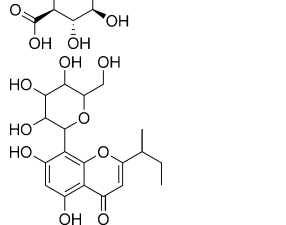
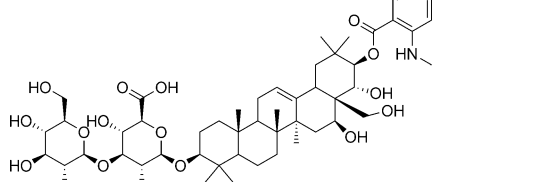
Here p is the expected screening hit rate for a given compound library and n is the total number of pooled compounds. The optimal pool size is defined as the maximum of the plotted curve. The observed screening hit rate for our natural product library was 0.15% which results in an optimal pool size of $n = 26$ (Figure S6). We successfully tested pools of up to 30 compounds, which included suramin as positive control (Figure S7). This makes it possible to screen more than 10000 compounds in a few hours.

Our screening revealed eight SIRT1 inhibitors (natural products 1–8; Table 1; Table S2), which were then tested for cytotoxicity in cell culture (Figure S8). Toxic compounds were excluded from further characterization. The IC_{50} values of the remaining five inhibitors were between 9.7 μM and 49 μM (Table 1). SIRT1 inhibitors with such potency can have valuable biological effects.^[24]

We thus tested the strongest inhibitor, **1** (Figure 4a), for its ability to increase p53 acetylation in human liver (HepG2) cells. Cells were treated for 16 h with 30 μM **1**. We observed a significant increase of p53 acetylation in the nuclear fraction by densitometric Western Blot analysis (Figure 4b).

We also used the described assay principle for the reverse reaction: acetylation of a substrate peptide by the acetyltransferase p300. Assay conditions were set at 0.5 μM of a p53-derived substrate peptide (HAT-peptide: STSRHKKL) and a degree of acetylation of about 35% (250 nM p300, 60 min; Figure S3 b) was chosen, such that either inhibition or activation of the enzyme could be detected.

Table 1: SIRT1 screening hits.

Structure ^[a]	IC ₅₀ [μM]
	9.7 ± 0.7
	28 ± 9
	36 ± 6
	46 ± 4
	49 ± 3
	n.d. ^[b]
	n.d. ^[b]
	n.d. ^[b]

[a] Structures were generated by using the program ChemDraw Ultra 9.0.
 [b] n.d.: not determined.

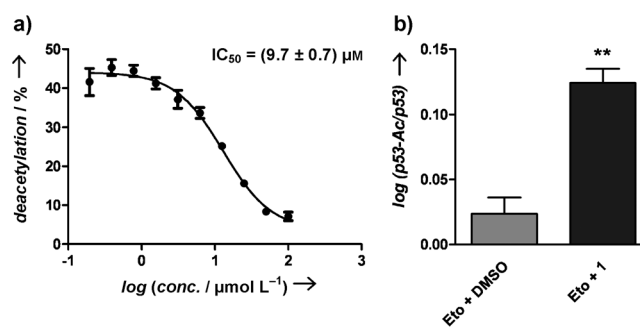


Figure 4. a) IC₅₀ curve for **1** and SIRT1 generated by MALDI quantification using H4 peptide (SGRGKGGKGLGKGGGA-K(Ac)-RHRK). b) Densitometric analysis of the Western blot data showing the effect of treating HepG2 cells with 30 μM **1** for 16 h on the acetylation state of p53 (Lys382) in the nuclear cell fraction (raw data: Figure S9). Basic p53 acetylation was induced by simultaneous treatment with 20 μM Etoposide (Eto).

We thereby discovered a novel p300 inhibitor (**9**; Figure 5 a; Table S2) and determined its IC₅₀ value to be (1.71 ± 0.07) μM (Figure 5 b). We confirmed this value by applying a radioactivity-based assay ((2.1 ± 0.4) μM). The quercetin derivative **9** is about 4 times stronger than anacardic acid

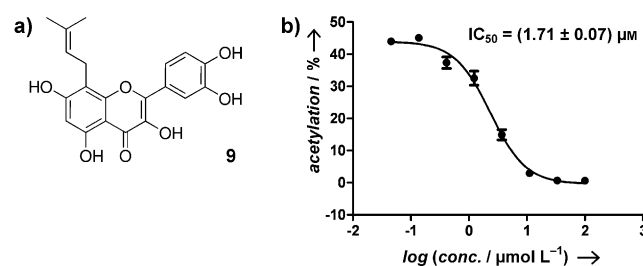


Figure 5. a) Structure of the new p300 inhibitor **9**. b) IC₅₀ curve for **9** and p300 generated by MALDI quantification.

(IC₅₀ = 8.5 μM), which is found in the shells of cashew nuts,^[25] and about 3.5 times stronger than garcinol (IC₅₀ = 7 μM), which was found in the fruit rind of a tropical evergreen tree.^[26] It is furthermore about 17 times stronger than quercetin, the core structure of **9**, which also inhibits p300^[27] but only showed an IC₅₀ around 34 μM in the radioactivity-based assay. In cell culture, **9** displayed significant anti-inflammatory effects by reducing the levels of TNFα secreted by THP-1 cells (Figure S10). This is an interesting feature of p300 inhibitors, which was recently shown for the p300 inhibitor curcumin.^[28]

The natural products described in this study may become valuable tools in the area of epigenetics and could in particular be used to shed more light on the role of histone deacetylases such as SIRT1 and acetyltransferases like p300 in various biological processes. Further in-depth studies are needed to explore their pharmacological profiles and potential health beneficial effects.

We have presented a straightforward MALDI MS based method for the unbiased screening and characterization of compounds that modify protein activity. Using this procedure

we could circumvent analytical problems deriving from compounds with autofluorescence. Clearly, a large variety of posttranslationally active enzymes like deacetylases, acetyltransferases, kinases, phosphatases, and methyltransferases can be studied in the presented way using appropriate substrate peptides and assay conditions.

Received: September 11, 2012

Revised: December 12, 2012

Published online: April 8, 2013

Keywords: compound screening · enzymes · MALDI · mass spectrometry · natural products

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