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Biomolecular-chemical Screening

A Novel Screening Approach for the Discovery of Biologically Active Secondary Metabolites

I. Screening Strategy and Validation†

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Chemical screening using thin-layer chromatography and various staining reagents offers the opportunity to visualize an almost complete picture of a microbial secondary metabolite pattern (metabolic finger-print). A thorough application of this strategy resulted in a number of biologically active new secondary metabolites, although the screening strategy is *per se* not correlated to any biological activity. In the present paper we report on a novel approach called biomolecular-chemical screening which combines the chemical screening strategy with binding studies of biological relevance. Making use of thin-layer chromatography (TLC) and subsequent staining, biomolecular-chemical screening allows to examine binding properties of low molecular weight metabolites to certain bio-macromolecules. The screening strategy itself, as well as independent validation of the results using DNA as selected bio-macromolecule are presented. The biomolecular-chemical screening method is useful to screen binding behaviour towards DNA of both, pure metabolites by one-dimensional TLC, and crude extracts by two-dimensional TLC. Investigation of pure secondary metabolites as well as screening of crude microbial extracts and new secondary metabolites obtained with this screening strategy are presented in accompanying papers.

In order to gain access to the outstanding molecular diversity of nature for drug development, various strategies like target-directed biological, physico-chemical, or chemical screening strategies are available[1~3]. Chemical screening applied to extracts from natural sources (*e.g.* microorganisms or plants) has already been proven to be an efficient supplemental and alternative method to target-directed screening programs. The chromatographic characteristics of metabolites on thin-layer chromatography (TLC) plates, as well as their chemical reactivity towards staining reagents under defined reaction conditions, allows to visualize an almost complete picture of a secondary metabolite pattern (metabolic finger-print)[4]. Using this analytical system, various new secondary metabolites have already been discovered in screening of microorganisms[3].

In contrast to biological screening, the chemical screening approach *a priori* is not correlated to a particular biological effect. The strategy, however, is based on the hypothesis that new structural classes of secondary metabolites most likely bear potential for interesting biological activities for future application[5]. Thus, chemical screening can be regarded as a systematic approach in the search for new biologically active compounds. The potential of the chemical screening strategy is to tap the outstanding structural resources from nature and to build a collection of pure natural products, new and known ones,

† Dedicated to Prof. Dr. HANS ZÄHNER on the occasion of his 70th birthday.

which then can be used for a broad biological testing[6]. Compound collections of substantial structural diversity contribute to improved lead discovery and efficiently complement synthetic libraries (*e.g.* from classical or combinatorial synthesis). Although reliable, fast, and efficient protocols for screening of extracts of micro-organisms like actinomycetes and fungi imperfecti have been established over the years, we decided to further develop the chemical screening approach. The present paper covers our recent results towards a new screening strategy the so-called biomolecular-chemical screening and its validation. Application studies, as well as physico-chemical properties and structure elucidation of new DNA-binding metabolites will be subject of accompanying papers[7,8].

Materials and Methods

General

To obtain random DNA, salmon sperm DNA (Sigma; 2 mg/ml in water) was homogenized by sonication for 6 minutes (Labsonic U, Braun). DNA-fragments were between 300 and 3,000 base pairs in size, as revealed by agarose gel electrophoresis. Denatured DNA was obtained by heating homogenized DNA at 95°C for 10 minutes and cooling on ice immediately after. Microbial extracts from 50 ml culture filtrate were prepared by absorption chromatography on Amberlite XAD-16 and elution with methanol - water (4 : 1) followed by a 50-fold concentration step. The protocol has been described already in detail[3,4].

TLC-screening

Biomolecular-chemical screening was performed on silica gel plates (Merck, HPTLC-ready-to-use-plates, silica gel RP-18 WF_{254S} on glass). All samples were spotted using the Automatic TLC Sampler III (Camag).

1D-TLC method: Pure substances dissolved in a suitable organic solvent like methanol, ethanol, acetone, chloroform or water in a concentration of 1 mg/ml (total amount per spot: 5 μg) were analysed for DNA-binding properties in a solvent system consisting of methanol - 1 M aqueous ammonium acetate solution (4 : 1). Homogenized salmon sperm DNA (total amount per spot: 4 μg) was spotted above the sample spots prior to chromatography.

2D-TLC method: Biomolecular-chemical screening was realized by two-dimensional TLC. Microbial extracts (total amount per spot: 5 μl of the 50 : 1 concentrated XAD-16 eluate) were separated in the first dimension using methanol - 0.5 M aqueous ammonium acetate solution (1 : 3). Interaction with DNA was analysed in the second dimension using methanol - 1 M aqueous ammonium acetate solution (4 : 1) as solvent system. DNA was spotted in a thin straight line above the separated extract with 5 μg DNA/cm before the second chromatographic step.

Detection was performed by means of UV extinction at 254 and 366 nm, as well as by colorization with staining reagents[9]. Changes in Rf-values indicate an interaction between ligand and DNA and are expressed by the Rf_2/Rf_1-ratio, in which Rf_1 represents the Rf-value without, and Rf_2 represents the Rf-value with DNA.

CD Spectroscopy

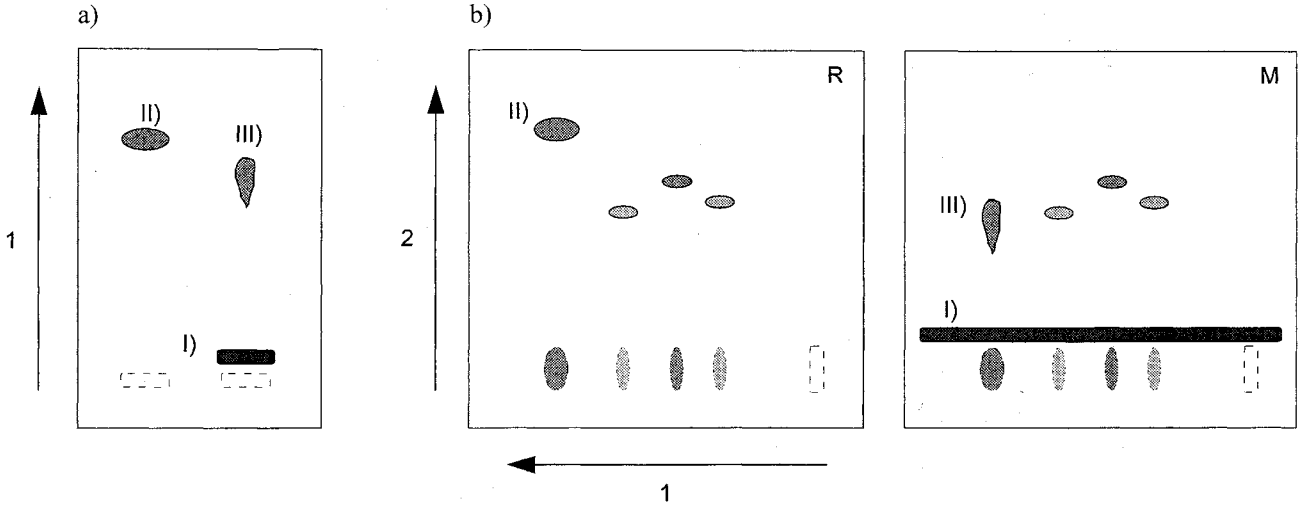
CD spectra of DNA were recorded on a Jasco Model 720 dichrograph using a 1 cm path length cell. All measurements were made in aqueous solution containing 200 mM ammonium acetate (corresponding to the solvent conditions in the TLC analysis) and DNA at an extinction of $E_{260}=0.6$. CD data are reported in $\Delta\varepsilon$ ($M^{-1}cm^{-1}$). The baseline was corrected for each solvent and ligand. The molar extinction coefficient was taken to be $\varepsilon_{260}=6{,}600$ $M^{-1}cm^{-1}$ for salmon sperm DNA.

To investigate the solvent influence on DNA conformation, samples were prepared as follows: homogenized DNA (2 mg/ml in water) was diluted with a 1 M aqueous stock solution of ammonium acetate and water to an extinction of $E_{260}=0.6$ in a final concentration of 200 mM ammonium acetate. Increasing methanol concentrations (v/v) were achieved by diluting DNA with required amounts of ammonium acetate, water and methanol. For the investigation of the solvent influence on the DNA-ligand complex, titration experiments with actinomycin D were carried out. Samples contained DNA in 200 mM ammonium acetate with or without 80% methanol as mentioned above. Concentration of actinomycin D is reported as total molar ratio (r') of ligand per nucleotide. All data were baseline and volume corrected.

Melting Curve Measurements

Melting profiles were measured using a Cary 1E UV-visible spectrophotometer (Varian) equipped with a thermostated cell holder. The temperature ramp rate was 1°C/minute from 20~98°C. Melting temperature (T_m) was calculated from the maximum of the first derivation of the temperature profile monitored at 260 nm. Samples of DNA-ligand interaction studies contained DNA (25 μg/ml) in 50 mM aqueous NaCl solution. Ligand was added in a total molar ratio of $r'=2$. Preparation of samples in aqueous ammonium acetate solutions with different methanol concentrations was carried out as described under CD-spectroscopy.

Fig. 1. Schematic depiction of TLC analysis.



a) 1D-TLC method for testing pure substances; left lane: chromatogram without DNA; right lane: chromatogram with DNA.
b) 2D-TLC method for screening extracts; M: measuring plate, R: reference plate.
I) Spotted DNA, II) Chromatogram without DNA interaction, III) Chromatogram with DNA interaction. Numbering of the arrows shows the sequence of TLC development. Solvent composition is given in the text.

Results

Screening Strategy

The concept of our biomolecular-chemical screening was to integrate chemical screening with target-directed screening approaches. Therefore, we combined the chemical analysis of secondary metabolite patterns by TLC with a binding analysis of low molecular weight compounds to bio-macromolecules. Our main objective was to take advantage of the high rate of new metabolites resulting from the TLC-based chemical screening and to perform an efficient binding analysis right on the TLC plate. To develop our method, random DNA was chosen as bio-macromolecule due to the number of well known DNA-binding molecules and the established knowledge on the interaction mechanisms. Moreover, new DNA-binding compounds are of ongoing interest for new antitumor drug development. It was our main focus to obtain biological binding data of both, pure secondary metabolites, and extracts of natural origin.

Method Development and Validation

For establishing the method of detecting compounds with affinity to DNA, the intercalating secondary metabolites actinomycin D, chelerythrine, doxorubicin, and nogalamycin were chosen as model compounds. Salmon sperm DNA was homogenized prior to use by ultra sonication to obtain DNA fragments between 300 and 3000 bp. For each binding study, two lanes were run on a TLC plate, pure ligand molecule as reference (left lane) and the ligand spotted together with DNA (right lane). After chromatography and visual detection the affinity of the tested compound to DNA was determined *via* the change in the Rf-value in comparison to the chromatogram without DNA (Fig. 1a). The affinity is expressed by the Rf_2/Rf_1-ratio, where Rf_1 represents the Rf-value without, and Rf_2 represents the Rf-value with DNA. It decreases significantly below 1 when interaction with the DNA occurs.

In order to find suitable conditions for binding analysis various types of TLC plates, solvent systems and detection methods (UV-detection at 254 or 366 nm, and staining reagents) were examined. Best results were obtained with RP-18 WF_{254S} silica gel plates using the solvent system methanol - 1 M aqueous ammonium acetate solution (4 : 1). In this solvent system, the spotted DNA remains at the starting point whereas the intercalating compounds as well as most low molecular weight natural substances exhibit Rf-values between 0.4 and 1. In addition, a physiological pH-value that is advantageous for the biological target could be maintained. The suitable amount of DNA, that provides sufficient free binding sites for DNA-binding

Table 1. Interaction between selected natural substances and DNA, detected by Rf-value-, and melting curve measurements.

Substance (5 μg)	Rf_2/Rf_1[a] 1 μg DNA	2 μg DNA	4 μg DNA[b]	10 μg DNA	ΔT_m [°C]
Actinomycin D	0.93	0.73	0	0	8.8
Chelerythrine	1	0.25	0	0	9.5
Doxorubicin	1	0	0	0	11.1
Nogalamycin	0.39	0	0	0	10.8
Erythromycin	1	1	1	1	0
Penicillin G	1	1	1	1	0
Glucose	1	1	1	1	0

[a] Changes in Rf-values are expressed by the Rf_2/Rf_1-ratio;
[b] 4 μg DNA is the determined standard amount for testing pure substances.

compounds, was examined by spotting increasing amounts while keeping a constant level of intercalators (see Table 1). It was found, that there exists an obvious dependence of ligand mobility on the amount of spotted target DNA as can be noticed by the decreasing value of the Rf_2/Rf_1-ratio at increasing amounts of DNA for each of the model compounds. A complete retention of the intercalators was observed by spotting 4 μg of homogenized DNA and 5 μg of ligand. To exclude unspecific binding effects, control experiments with non-binding compounds were carried out. Even with 10 μg of DNA no effect on the Rf-values was observed for structurally diverse compounds as erythromycin, penicillin G, or glucose. No retention of the intercalating model compounds was found using denatured target DNA that lacks the original double helical conformation (data not shown). That clearly showed that binding of low-molecular weight compounds to DNA on the TLC plates occurs in a specific manner.

The results obtained on TLC plates were confirmed by comparative thermal melting analysis of the random DNA and DNA-ligand complexes. Non-binding substances caused no change in the melting temperature (T_m) of DNA whereas the binding ligands stabilized the DNA and therefore caused a significant increase in T_m (see Table 1).

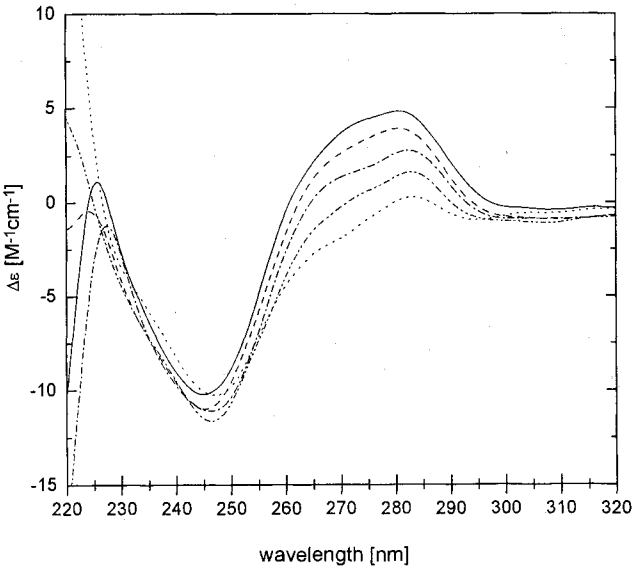
Biophysical Investigations on the Target DNA

In order to study the structural integrity of the homogenized salmon sperm DNA in the TLC solvent system (80% methanol in ammonium acetate), CD analysis was carried out. In the presence of increasing methanol concentrations the CD spectra of the DNA exhibited a significant decrease of the positive CD couplet at 283 nm (Fig. 2), which correlates with a decrease in thermal stability of the DNA (Table 2). These data indicate a transition from the native B-conformation of DNA to a more compact form resembling the C-conformation under high salt conditions[10]. Actinomycin D as a potent intercalator was then selected for a more detailed characterization of DNA binding properties under the influence of the solvent system used for the TLC method. Binding of actinomycin D to DNA causes conformational changes and therefore can also be monitored by CD spectroscopy. In titration experiments with increasing amounts of actinomycin D at a constant level of homogenized DNA, the CD couplet at 283 nm progressively increased and reached a saturation level at room temperature for $r'=0.4$, which is the total molar ratio of actinomycin D to nucleotide (Fig. 3). DNA in 80% methanol exhibited a lower affinity towards the intercalator, as indicated by the more moderate slope of the binding curve between $r'=0$ and $r'=0.2$. The CD binding effect measured in the solvent system used in the TLC experiments showed only a slight decrease from $\Delta\varepsilon=1.67$ $M^{-1}cm^{-1}$ to $\Delta\varepsilon=1.45\,M^{-1}cm^{-1}$ indicating that binding of actinomycin D still occurs. The above findings suggest that the target DNA is not denatured under the organic solvent

Table 2. Melting temperatures of homogenized salmon sperm DNA in 200 mM ammonium acetate in dependence on increasing methanol concentrations.

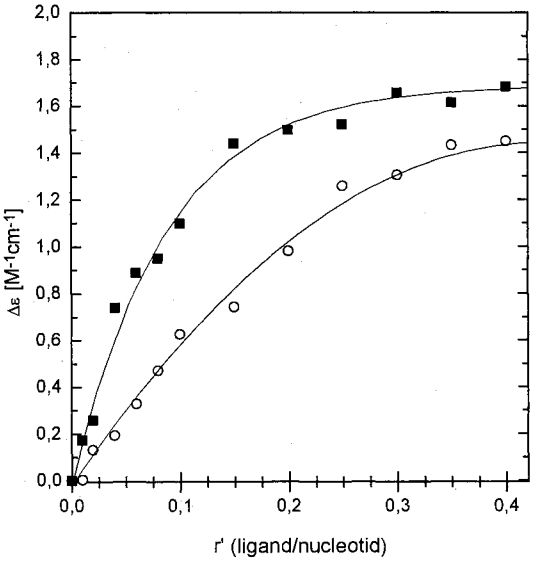
	T_m [°C]					
	Methanol concentration (v/v)					
	0 %	20 %	40 %	60 %	70 %	80 %
Homogenized DNA	90.9	81.7	72.6	62.1	55.9	40.6

Fig. 2.



CD-spectra of homogenized salmon sperm DNA in 200 mM aqueous ammonium acetate with increasing methanol concentrations (v/v): (—) 0% methanol; (---) 20% methanol; (-·-) 40% methanol; (-···) 60% methanol; (···) 80% methanol.

Fig. 3.



CD titration curves of homogenized salmon sperm DNA with actinomycin D in 200 mM aqueous ammonium acetate containing different methanol concentrations (v/v) monitored at 283 nm: (■) 0% methanol; (○) 80% methanol.

influence and binding of ligands is not disturbed. On the basis of these studies, validation of the developed method to study binding of low molecular weight compounds to DNA on TLC has been achieved. Thus, it is possible to apply this method to the examination of various pure natural products towards DNA-binding properties.

Biomolecular-chemical Screening

For binding studies with secondary metabolites from complex crude microbial extracts, allowing an application as a screening method, a two-dimensional TLC system was worked out. In the first dimension the separation of the individual metabolites of the crude sample was achieved, followed by the analysis of the binding properties of the separated spots in a further chromatographic step in the second dimension (Fig. 1b). As in the studies on pure compounds, DNA-binding is indicated after chromatography with and without DNA through a decrease of the Rf_2/Rf_1-ratio significantly below 1.

The Rf-values of many secondary metabolites on RP-18 WF_{254S} silica gel plates were above 0.7 in the solvent

system used for one-dimensional TLC binding studies (methanol - 1 M aqueous ammonium acetate solution=4 : 1). In order to enlarge the chromatographic window for the separation of the metabolites in the crude extracts in the first dimension of the TLC, the solvent system was changed to methanol - 0.5 M aqueous ammonium acetate solution (1 : 3). After this initial separation step homogenized DNA was spotted on the TLC plate as depicted in Fig. 1b. Based on the optimization of the solvent system for one-dimensional binding studies, methanol - 1 M aqueous ammonium acetate solution (4 : 1) was used for the second chromatographic step. First screening attempts resulted in isolation and structural characterization of both, known compounds like phencomycin[11], menoxymycin B[12], or soyasaponin I[13] and several new secondary metabolites[8].

Discussion

Determination of binding of low molecular weight compounds to DNA by one-dimensional TLC is an easy-to-handle method which allows an efficient and reliable analysis with pure compounds in parallel. Because visualization is achieved either by the color of a compound, its UV-extinction, or with various staining reagents, the method is very flexible towards the type of compound to be analysed. We consider the TLC-based method to be very useful for various applications. On the one hand, as an analytical tool it can be used in chemical synthesis to control purity and affinity of substances to DNA in one step. On the other hand, the method can be utilized as a pre-screen for more detailed binding studies using viscosimetric methods, CD-, X-ray-, or NMR-analysis. In this context it seems possible to employ the method for analysis of sequence specificity of DNA-intercalating agents. At present, this is limited by the large amount of DNA necessary for the TLC studies. Thus, miniaturization should be a critical objective for further method development. A more detailed investigation on a number of structurally diverse secondary metabolites from microbial sources using the presented method will be given in the accompanying paper[7].

First attempts in using other bio-macromolecules, *e.g.* proteins like the estrogen-binding protein of *Candida albicans*[14], turned out to be not successful. Proteins tend to lose their biological activity under the solvent conditions used in TLC, that are necessary for the chromatography of most natural compounds. Up to now, we did not succeed to find a suitable analytical TLC system.

The biomolecular-chemical screening performed with DNA as bio-macromolecular target using two-dimensional TLC is a novel and interesting supplement of the standard chemical screening. Advantageously, chemical screening with its possibilitiy to determine a metabolic finger-print of secondary metabolites and its high success rate in the discovery of new compounds was combined with a biological binding analysis. A limitation is the lower chromatographic resolution on RP-18 WF_{254S}-silica gel plates in the separation step in comparison to $60F_{254}$-silica gel plates, commonly used in our standardized chemical screening program, or HPLC techniques. In addition, chromatography in the second dimension (binding analysis) resulted in further spot-broadening. However, by using RP-18 WF_{254S}-silica gel plates and methanol-aqueous ammonium acetate mixtures as solvent systems, a physiological pH-value concerning the biological target, and reasonable Rf-value resolutions were achieved. In consequence, the information on affinity to DNA adds useful selection criteria to the standard chemical screening. This allows to identify new secondary metabolites with DNA-binding properties in complex mixtures, which often is not possible with other screening methods.

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