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Identification of adenosine A1 receptor ligands from *Morus alba* L. stem bark by NMR metabolomics approach

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Abstract

Comprehensive extraction coupled to NMR metabolomics was applied to the identification of active compounds from *Morus alba* stem bark binding to the adenosine A1 receptor. Orthogonal projection to the least square (OPLS) analysis was used to estimate which compounds significantly correlate with the activity. Based on the loading bi-plot and Y-related coefficient plot, unsaturated fatty acid signals strongly correlate to the activity. The characteristic NMR signals of alkaloids and prenylated aromatic compounds found to be abundant in this plant do not positively correlate with the activity. Characteristic signals from two compounds isolated from this plant (betulinic acid and morusin) also have negative Y-related coefficients and indeed they are inactive when tested. Aromatic compounds without prenyl or methoxy units are proposed to be the active ones. To further identify the possible active compounds, the comprehensive extraction needs to be optimized, particularly on the selection of a solvents combination which gives a high resolution of the metabolites over the fractions.

Introduction

Mullberry or *Morus alba* L. (Moraceae) is cultivated mainly for its foliages as silkworms feed (Isabelle *et al.*, 2008), but in several European countries such as Greece and Turkey the fruits are also used as a table fruit, marmalade, juice, liquor, and as natural dyes (for wool or cotton) (Gerasopoulos and Stavroulakis, 1997). In traditional Chinese medicine, different parts of this plant are used to cure various diseases such as diabetes (leaves, stem and root bark), tonic, diuretic and high blood pressure (fruit) (Isabelle *et al*., 2008).

Numerous compounds have been isolated from *M. alba* including sugar-mimic alkaloids, polyhydroxylated alkaloids, prenylated chalcones and flavonoids, stilbenes, and several compounds having benzofuran skeleton with prenyl or methoxyl groups attached to it. These compounds were reported to have diverse bioactivities such as glycosidase inhibitors, human cancer cells proliferation inhibitors, antiviral and antimicrobial, antiasthmatic, and antioxidant activities (Nomura *et al*., 1978; Nomura *et al.*, 1983; Asano *et al*., 2001; Doi *et al*., 2001; Du *et al*., 2003; Piao *et al.*, 2009; Zhang *et al.*, 2009a; Zhang *et al*., 2009b; Tian *et al*., 2010; Yang *et al.*, 2010a; Fu *et al.*, 2010).

In this research we used the adenosine A1 receptor as a screening method. It was previously reported that adenosine A1 receptor antagonist can induce diuresis and sodium excretion (Modlinger *et al*., 2003). Indeed the receptors are expressed in different parts of the kidney, including in the afferent arterioles, glomerulus, proximal tubules and collecting ducts. The mechanism of adenosine antagonists on sodium and water excretion may be based on direct inhibition of sodium re-absorption in proximal tubules, or indirectly by promoting afferent arteriole dilatation (Poulsen *et al*., 1998; Modlinger *et al*., 2003). Flavonoids and xanthine derivatives are the two natural compound groups reported to have antagonistic binding activity to the adenosine A1 receptor (Ingkaninan *et al*., 2000; Alexander, 2006; Yuliana *et al.*, 2009).

We have recently highlighted the benefits of the use of metabolomics as an alternative tool to identify bioactive compounds from medicinal plants (Yuliana *et al*., 2011; Yuliana *et al*., 2012). In the present study we used a similar approach to identify compounds from *M. alba* stem bark showing binding activity to the adenosine A1 receptor. We applied a new extraction method we recently developed to provide several fractions of *M. alba* stem bark which contain fewer overlapping compounds as compared with

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fractions from single solvent extraction. Before the extraction, a de-fatting step by n-hexane extraction was used to remove unsaturated fatty acids as they bind unspecifically to this receptor. The obtained fractions were then tested for the adenosine A1 receptor binding activity and the metabolite profile was measured by NMR. Orthogonal partial least square (OPLS) analysis with proper validations was used to check which compounds correlate with the adenosine A1 binding activity of the plant. Positive correlations between specific NMR signals for particular compounds present in *M. alba* extract and their binding activity to the adenosine A1 receptor will give scientific support for traditional use of this plant extract as diuretic agent.

Materials and Methods

Chemicals and reagents

Methanol, n-hexane, acetone, ethyl acetate, HCl, NaOH, and DMSO were purchased from Biosolve BV (Valkenswaard, The Netherlands). Tris buffer was purchased from Gibco BRL (New York, NY, USA). [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was from DuPont NEN, and CPA (N6-cyclopentyladenosine) was from RBI Inc. (Zwijndrecht, The Netherlands). Kieselguhr (calcined and purified SiO_2) was bought from Fluka Analytical/ Sigma Aldrich Chemie GmbH (Steinheim, Germany). All solvents and reagents were of analytical grade.

Plant material

Morus alba stem bark was obtained from Korean Export and Import Federation of Drugs, Seoul, South Korea and identified by Dr. Young Hae Choi (Leiden University).

Comprehensive extraction

Firstly, very non-polar lipids were removed from the powdered plant material before the comprehensive extraction starts by adding 3 mL of n-hexane to powdered plant material, followed by sonication for 15 minutes, and then the solvent was removed. This was repeated 2 times with 2 mL of n-hexane. The plant material was dried under N_2, mixed with Kieselguhr, and loaded into the extraction column. The combination of solvents used for the comprehensive extraction was ethyl acetate-methanol (1:1) as solvent A, and methanol-water (1:1) as solvent B. The flow rate was 4 mL min^{-1} and fractions were collected every two minutes. Thirty fractions of 8 mL each were obtained at the end of the extraction. One and half mL of the extract was sampled from each fraction to be used in the bioassay. The remaining volume (6.5 mL) was used for NMR measurement where methanol-d4 was used as a solvent. The extraction was performed in three replicates. The extraction scheme can be seen in Table 1.

Table 1. Extraction scheme: T = time (min), A = ethyl acetate-methanol (1:1), B = methanol-water (1:1). The flow rate was 4 mL min^{-1}

T (min)	Gradient
0 – 10	100% solvent A
10 – 50	100% solvent A to 100% solvent B
50 – 60	100% solvent B

NMR measurement

NMR measurement was performed according to the method described elsewhere (Kim *et al.*, 2010).

Data analysis

The ^{1}H NMR spectra were automatically reduced to ASCII files. Bucketing was performed by AMIX software (Bruker). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3–10.0. The regions of δ 4.75–4.9 and δ 3.28–3.34 were excluded from the analysis because of the residual signal of H_2O and methanol *d*-4, respectively. Orthogonal partial least square analysis (OPLS) was performed with the SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden) with scaling based on the Pareto method.

Adenosine A1 receptor assay

The assay was performed as previously described (Chang *et al*., 2004) except that the volume of the total mixture in the assay was modified from 400 µL to 200 µL. The radioactive ligand used for the assay was 0.4 nM [^{3}H] DCPCX (8-cyclopentyl-1,3-dipropylxanthine) (Kd = 1.6 nM). Membranes were prepared from Chinese hamster ovary (CHO) cells stably expressing human adenosine receptors. CPA (N6-cyclopentyladenosine) was used to determine non-specific binding. The mixture consisting of 50 µL [^{3}H]DPCPX, 50 µL CPA/50 mM Tris-HCl buffer/test compounds in different concentrations, 50 µL 50mM Tris-HCl buffer pH 7.4, and 50 µL of membrane was incubated at 25°C for 60 min and then filtered over a GF/B Whatman filter under reduced pressure. The filters were washed three times with 2 mL ice-cold 50 mM Tris/HCl buffer, pH 7.4, and 3.5 mL scintillation liquid was added to each filter. The radioactivity of the washed filters was counted by a Hewlett-Packard Tri-Carb 1500 liquid scintillation detector. Non-specific binding was determined in the presence of 10–5 M CPA. The bioactivity was described as percentage of inhibition of [^{3}H]DPCPX binding to the adenosine A1 receptor by the extracts and was calculated by using

the software package Graphpad Prism (Graphpad Software Inc., San Diego,CA, USA).

Results and Discussion

The binding activity profile of *M. alba* fractions is presented in Figure 1. The activity was concentrated in the first 10 fractions. This is confirmed further with the OPLS score plot which shows that these 10 fractions are separated from the inactive ones (Figure 2A). From the loading bi-plot, some signals in the aliphatic (1) and aromatic (2) region are found to be abundant in the active fractions (Figure 2B).

The model was then statistically validated. From the external validation (in which the data were divided into two: calibration group and test group), the value of root-mean-square errors of prediction (RMSEP) is higher than root-mean-square errors of estimation (RMSEE) indicating a slight overfit. The R2Y and Q2Y values after cross validation are close to 1 (0.90 and 0.88 respectively) and the p value from ANOVA cross-validation is relatively small ($2.23.\ 10^{-36}$), showing that the model is statistically acceptable.

As can be seen in the OPLS Y-related coefficient plot (Figure 3), most of aliphatic and aromatic signals have a positive correlation, while most signals between δ 2.40 – 5.16 have a negative correlation. Unsaturated fatty acid signals (δ 1.28 – 1.36) are shown to have the highest positive correlation to the activity. Apparently the de-fattening steps performed before the extraction did not successfully remove these false positive compounds from the plant material.

From previous work in our lab we also isolated betulinic acid and morusin from *M. alba* stem bark. Both compounds do not show any binding activity to adenosine A1 receptor. Here we checked the correlation of signals present in these two compounds to its adenosine A1 binding activity based on the value of Y-related coefficient from the generated OPLS model (Figure 3).

Betulinic acid is a pentacyclic triterpenoid. Although some of its signals are positive, e.g. methyl protons of C-23 – C-27 (located between δ 0.70 – 1.00), but some signals are located in negative part of the plot, such as two vinyl proton signals at C-29 which are located between 4.50 – 4.60 ppm. Morusin is a prenylated flavonoid, a typical class of compounds found in Moraceae. It has a flavone skeleton with a prenyl group attached to C-3 and a 2,2-dimethylchromene ring connected to A ring. Similar to betulinic acid, some signals of morusin are located in the positive area and some are in the negative area of the Y-related coefficient plot. Signals

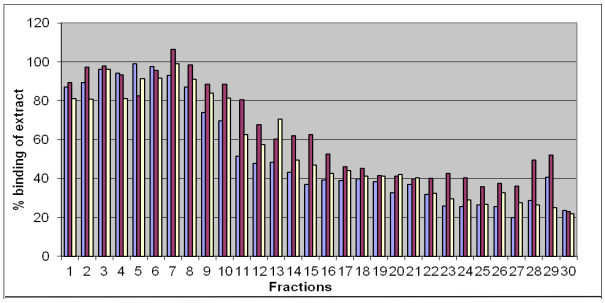

Figure 1. The adenosine A1 binding activity of *Morus alba* fractions obtained from comprehensive extraction. Extraction was performed in three replications (represented by different bar colours). Each replication resulted in 30 fractions. Bioactivity test was performed for each fractions in duplo.

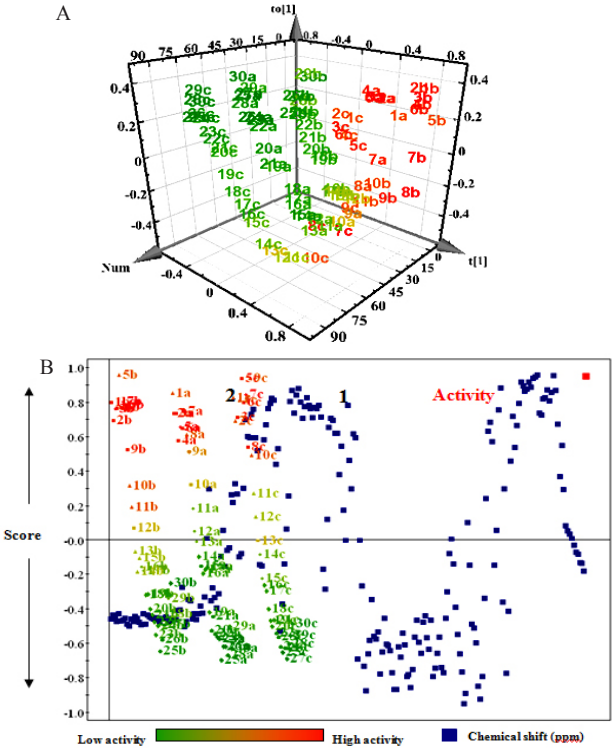

Figure 2(A). The predictive and the first orthogonal component of *M. alba* OPLS scores scatter plot, fractions are labeled by number with a-c representing replications. R^2X=48.3%, $R^2XOrthogonal_1$=30.2% (B). The loading bi-plot: 1= aliphatic region, 2 = aromatic region. Chemical shift with a score value similar to certain fractions are abundant in the respective fraction(s).

of methyl protons of the 2,2-dimethylchromene ring, which are located between δ 1.40 – 1.50 (overlap), are positively correlated with the activity. Some signals from aromatic protons of the flavone skeleton (δ 6.00 – 7.00) are located in the positive coefficient area but few signals are negative. Signals from methylene and vinyl methine protons of the prenyl unit (between δ 3.00 – 5.50) are located in the negative area.

It has to be noted that certain signals of different compounds may overlap. This may result in a positive correlation with the activity of a signal of

Table 2. Statistical validation of *M. alba* OPLS model

R^2Y	0.90
Q2Y	0.88
p CV-ANOVA	$2.23.\ 10^{-36}$
RMSEP	12.04
RMSEE	9.10

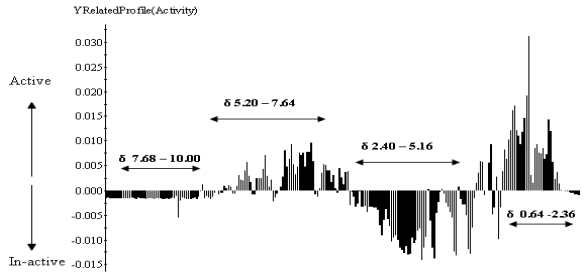

Figure 3. The Y-related coefficient plot

non-active compounds, or the reversed. Reducing the bucket size of NMR data from 0.04 into 0.02, 0.01, and 0.001 ppm did not solve the problem. The proton NMR spectra of *M. alba* fractions obtained from comprehensive extraction indeed do not show a good resolution, which can be seen in many overlapping peaks especially in the first three fractions. However, as most compounds have several signals still the active compounds may be traced by this approach. Though, further optimization of the comprehensive extraction should be considered. Particularly, attention should be given to the selection of a solvents combination which gives a high resolution of the metabolites over the fractions.

However, we further tried to elucidate the possible active compounds. We chose fraction 7b as the most active fraction for 2D NMR analysis. There are several correlations on COSY and HMBC spectra which can be attributed to the skeletons of flavone, flavane, and prenyl units. Unsaturated fatty acids signals are predominant here which can be the reason for the high activity. As previously described, several classes of compounds which have aromatic and non-aromatic structures have been isolated from *M. alba* (Nomura *et al.*, 1978; Nomura *et al*., 1983; Asano *et al.*, 2001; Doi *et al.*, 2001; Du *et al.*, 2003; Piao *et al.*, 2009; Zhang *et al.*, 2009a; Zhang *et al*., 2009b; Tian *et al*., 2010; Yang *et al.*, 2010a; Fu *et al.*, 2010). From the activity profile, it is clear that the activity cannot be attributed to alkaloids which are abundant in polar fractions. Besides, the areas where alkaloid characteristic signals are located have a negative correlation with the activity. Based on the Y-related coefficient plot, the active principals could be the compounds which have aromatic ring without any groups containing protons which give signals in δ 3.00 – 5.00 (beside alkaloids and sugars, signals within this range can be also from protons of vinyl, methine or methylene groups such as prenyl, geranyl, or farnesyl groups, or methoxy group). This is in accordance with the result from previous work which concluded that compounds isolated from Morus species having more complex ring systems due to the prenylation, have stronger cytotoxic activity (Yang *et al.*, 2010a), and that the prenylation itself is not important for the anti-viral activity (Du *et al*., 2003). Moracin E, moracin M, and moracin X which have a benzofuran ring can be one of them, but not for example moracin A, B, C, F, G, H, N, P, V, W and Y since in the latter group all contain methoxy, formoyl, prenyl or dihydrooxepin substituents and these have signals in the negative area. However, this prediction should be validated further by testing the above mentioned compounds with the respected bioactivity.

Conclusion

Comprehensive extraction integrated with NMR metabolomics is able to identify the correlation of compounds found in *M. alba* stem bark with their adenosine A1 binding activity. Unsaturated fatty acids are found to be strongly correlated with the activity, while alkaloids and prenylated aromatic compounds are predicted not to be active. The chemical validation by using two inactive compounds isolated from this plant showed that characteristic signals of these two compounds indeed have negative Y-related coefficients. The information obtained from the loading bi-plot and Y-related coefficient plot shows that the active ones should have aromatic structures without any methoxy or prenyl groups attachment. The approach as described here allows to exclude certain compounds for being involved in the activity. By 2D NMR further information on the spin systems of activity related signals can be obtained. This might already result in the identification of the active compound(s). If full structure elucidation of a novel compound is required, isolation will be necessary which also enables final testing of the biological testing. In this approach, no bioasssay-guided fractionation is applied, instead fractionation will be monitored on the basis of the targeted signals. This approach is particularly of interest for in combination with complex *in-vivo* bioactivity assays, including clinical trials of medicinal plants.

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