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*Original Article*

# Pau d'arco activates Nrf2-dependent gene expression via the MEK/ERK-pathway

**Michelle Richter, Angelika F. Winkel, Dietmar Schummer, Martin Gerlitz, Meltsje de Hoop, Bodo Brunner, Maike Glien and Dieter Schmoll**

*Sanofi-Aventis Deutschland, R&D, Industriepark Hoechst, D-65926 Frankfurt, Germany*

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**ABSTRACT** — Pau d'arco is a plant-derived traditional medicine that acts by poorly understood molecular mechanisms. Here, we studied the effect of pau d'arco on the cytoprotective transcription factor Nrf2. An aqueous extract of pau d'arco stimulated Nrf2-dependent gene expression and led to nuclear localization of Nrf2 *in vitro*. Chromatographic separation and mass spectrometry of the extract identified benzene trioles or benzene tetraoles within the active fractions. The extract stimulated the mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK1/2) pathway. The pharmacological inhibition of MEK, but not of p38 mitogen-activated protein kinase, glycogen synthase kinase-3 or phosphoinositide 3-kinase was required for the activation of Nrf2-dependent gene expression by pau d'arco, but not for the nuclear translocation of Nrf2. *In vivo* pau d'arco increased the expression of Nrf2-target genes in the intestine. The results suggest that the activation of Nrf2 could mediate beneficial effects of pau d'arco, in particular in the intestine.

**Key words:** Red lapacho, Nrf2, ERK, Keap1, Phytomedicine

## INTRODUCTION

Pau d'arco, also called red lapacho, is an extract from the inner bark of *Tabebuia impetiginosa* and is traditionally used as a botanical drug in particular in South America for its immune-strengthening properties (Gómez Castellanos *et al.*, 2009). It has also been claimed to be beneficial for cancer patients, which could depend on the targeting of topoisomerase 1 (Gómez Castellanos *et al.*, 2009; Li *et al.*, 1993). The extract consists of many different chemicals, including quinoids and flavonoids and much of the research addressing the biologic action of pau d'arco has focussed on lapachol, a naphthoquinone, and quercetin, a flavonoid, as active ingredients (Gómez Castellanos *et al.*, 2009). The molecular mechanisms mediating the anti-inflammatory and anti-microbial effects of pau d'arco have not been characterized in detail.

The transcription factor nuclear factor erythroid 2 p45 (NF-E2)-related factor (Nrf2) is critical for the expression of several cytoprotective genes, including NAD(P)H:quinone-oxidoreductase-1 (NQO1) and heme oxygenase 1 (HMOX1) (Dinkova-Kostova and Talalay, 2010). Nrf2 is regulated by the adaptor protein Keap1, which mediates the ubiquitinylation and degradation of Nrf2 (reviewed in Copple, 2012). Keap1 is inhibited by the electrophilic or oxidative modification of several of its cysteine residues (Yamamoto *et al.*, 2008). This prevents the degradation of Nrf2 and results in the nuclear translocation of the transcription factor, where it binds as a heterodimer with small Maf proteins to the so-called antioxidant response element (ARE), the promoter region of its target genes. Protein kinases such as extracellular-signal-regulated kinase (ERK), p38 mitogen-actived protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) and glycogen synthase kinase-3 (GSK3) can also modulate the activity of Nrf2 (Copple, 2012).

The activation of Nrf2 triggers a pleiotropic biological response that increases cellular stress resistance and has drawn high medical interest as a potential approach for the prevention and treatment of diseases including cancer, neurodegenerative, cardiovascular, metabolic, and inflammatory diseases. Pharmacological activators of Nrf2 include the triterpenoid bardoxolone-methyl that was in late-stage clinical development for the treatment of chronic kidney disease (Pergola *et al.*, 2011) and sulforaphane,

Correspondence: Dieter Schmoll (E-mail: dieter.schmoll@sanofi.com)

an isothiocyanate derived from broccoli that shows anti-inflammatory and anti-oncogenic properties in preclinical models (Dinkova-Kostova and Kostov, 2012). As several phytomedicines have been linked with Nrf2 signalling, we studied whether this pathway could also be a molecular target of pau d'arco.

## MATERIAL AND METHODS

### Preparation of aqueous pau d'arco extract

The aqueous extract was prepared from two different sources of pau d'arco. In most experiments an extract was used where one tablet of pau d'arco (Nature's Own, Sanofi Consumer Healthcare, Australia) that contained 500 mg inner stem bark of *Tabebuia avellanedae* was extracted overnight in 2 ml water at room temperature. The solution was cleared by centrifuging and the supernatant was used in the subsequent experiments. A concentration of 0.5% (v/v) of pau d'arco relates to a 1:200 dilution of this extract. In addition, an aqueous extract was prepared by extracting 250 mg of pau d'arco tea (Schoeneberger, Magstadt, Germany) for 3 hr at 95°C in 1 ml water followed by centrifuging as described above.

### Cell culture

HepG2 cells were cultivated in MEM GlutaMAX (Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (PAA) and 1% non-essential amino acids (Life Technologies), at 37°C and 5% $CO_2$ in a humidified incubator. Cells were kept subconfluent and diluted twice a week.

### Generation of the Nrf2-Reporter gene cell line and reporter-gene assay

Into the pGL3 Luciferase Reporter Vector (Promega, Mannheim, Germany) containing the neomycin-resistance cassette, an oligonucleotide with the sequence of eight ARE was cloned upstream of the SV40 promoter as described (Wang *et al.*, 2006). HepG2 cells were transfected with this construct and selected for neomycin resistance. The clone was propagated based on the ability of described Nrf2 activators such as sulforaphane to induce reporter-gene expression in an Nrf2-dependent manner as demonstrated by siRNA experiments (data not shown). After an incubation of 16-20 hr with varying concentrations of pau d'arco, reporter-gene assays were performed using the Bright Glo luciferase assay system (Promega).

### RNA interference

HepG2 cells were reversely transfected with either a non-silencing small-interfering RNA (siRNA) or with siRNAs targeting Nrf2 expression, namely #SASI_Hs01_00182393 (Sigma-Aldrich, St. Louis, MO, USA) (siNRF2, A) or #SI03246950 (Qiagen Ltd., Hilden, Germany) (siNRF2, B) using Lipofectamine RNAiMAX (Life Technologies). Therefore, per well of a collagen-treated 96-well plate 0.2 µl Lipofectamine RNAiMAX and 5 µM siRNA were diluted in a final volume of 20 µl OptiMEM (Life Technologies) and incubated for 15 min at room temperature. Subsequently, 15,000 HepG2 cells were added per well reaching a final siRNA concentration of 50 nM. 24 hr after transfection, pau d'arco was added to the wells. Analysis of luciferase activity and gene expression were performed after a subsequent incubation of 20 hr.

### Reverse transcription and real time PCR

Total RNA was isolated by using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription and PCR were carried out using the High Capacity cDNA Reverse Transcription Kit (#436881, Life Technologies) and the Taqman Gene Expression Master Mix Kit (#4369016, Life Technologies), respectively. As human primers were used: Hs 00157965-m1 (HMOX1), Hs 01045994-m1, and Hs 01102345-m1 (RPL37a); mouse primers were Mm 0051006-m1 (HMOX1), Mm 00500821-m1 (NQO1) and Mm 01546394-s1 (RPL37a), all from Life Technologies. Quantification was performed with a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using the δδCt method with RPL37a as internal standard.

### Nrf2-antioxidant Response Element (ARE) binding activity

The Nrf2-ARE binding activity in nuclear extracts was measured using the Trans AM Nrf2 Kit (Active Motif, Carlsbad, CA, USA). The preparation of nuclear extracts was described previously (Schmoll *et al.*, 1999, 2001). Ten µg of nuclear protein were incubated in 96-well plates pre-coated with ARE consensus oligonucleotides (5`-GTCACAGTACTCAGCAGAATCTG-3`) according to the manufacturer's protocol. Absorbance was measured using a plate reader at 450 nm after 15 min. The specificity of binding was determined by the competition with oligonucleotides of either the wild-type ARE consensus sequence or a mutated sequence according to the manufacturer's instructions. Results were expressed as the difference in absorbance measured in the presence of the mutated oligonucleotide and the competing probe.

### Confocal laser scanning fluorescence microscopy

Approximately 8,000 HepG2 cells were seeded onto collagen R coated leaves (Ibidi, Martinsried, Germany). On the next day the cells were transfected with 2 µg of plasmid pcDNA 3.1.-HA-Nrf2, expressing N-terminal HA-tagged human Nrf2 under the CMV promoter, using X-tremeGENE 6 DNA transfection reagent (Roche Diagnostics) at a ratio of 2:6 (X-tremeGENE 6 (µl) to DNA (µg)). 72 hr after transfection, the cells were incubated for 2 hr with or without pau d'arco and the MEK inhibitors as indicated. Cells were fixated with 0.5 ml of 4% formaldehyde in PBS for 30 min at 22°C and subsequently rinsed twice for 10 min with PBS containing 0.2% (w/v) Triton X-100 (PBS-T) for permeabilization. Immunofluorescence was performed using anti-Nrf2 antibody H300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS-T containing 3% (w/v) BSA overnight at 4°C. After washing three times with PBS-T, samples were incubated with an Alexa Fluor 488-labelled anti-rabbit IgG antibody (Life Technologies) for 2 hr at 37°C followed by washing with PBS-T. In order to stain the nuclei, the cells were incubated for 1 hr at 4°C in 0.5 µM To-Pro-3 Iodide (Life Technologies) and then washed three times with PBS. Confocal laser scanning microscopy was carried out using a DM IRE2 (Leica, Wetzlar, Germany) at excitation and emission wavelengths of 536 and 617 nm for red fluorescence and at 485 and 530 nm for green fluorescence.

### Animal studies

Vehicle (PBS, 1% Cremophor-EL and 1% DMSO) or pau d'arco (5,000 mg/kg) were administered by oral gavage to female C57Bl/6NCrl mice twice with the second application 12 hr after the first. Animals were killed after additional 5 hr and tissues were dissected. In order to isolate intestinal cells, guts were turned inside out and cells scraped of the basal membrane. Samples were stored in RNAlater (Qiagen). RNA was isolated using the Qiagen RNA kit. Gene expression was quantified as described above. All experimental procedures were conducted in accordance with the German Animal Protection Law and adhered to the international animal welfare legislation and rules.

### Chromatographic separation and analysis of the pau d'arco extract

20 µl of re-dissolved extract were separated on an analytical RP18 HPLC column (Phenomenex Luna 3 µm C18, 150 x 3 mm, Phenomenex, Torrance, CA, USA; gradient 5-95% acetonitril). The eluent was collected into 88 fractions (3 fractions/min), fractions were dried and the biological activity was tested. In parallel to the fractionation, the HPLC separation was detected by UV (DAD, Agilent HPLC 1100, Agilent, Santa Clara, CA, USA) and mass spectrometry (Micromass LCT, ESI-TOF).

The MS-data were recorded and analyzed by MassLynx-version 4.0 software (Waters, Milford, MA, USA) and brought into correlation with the collected fractions used for biological screening.

Detection of MS-trace (total ion count) was performed by recording positive ions and in the following run by recording negative ions by Micromass-TOF using orthogonal electrospray-ionization and lock-spray. A sampling rate of 0.5 Hz and a detection limit of 200-1,500 amu was used for recording mass spectra.

Active fractions were redissolved and analysed by UHPLC (Kinetex 1.7 µ C18 100 A, 50 x 2, 1 mm, Phenomenex; gradient 5-95% acetonitril); UV (DAD) and HR-MS (Agilent HPLC 1200 and Agilent 6220, ESI-TOF) detection. Identified peaks were characterized by UV spectra, mass spectra, and molecular formula (calculated from accurate mass and isotope pattern), and data matched against the corresponding data of literature-known natural products (Dictionary of Natural Products, Buckingham, 2010).

### Statistical analysis

All data are presented as the mean ± S.E.M. unless indicated differently. Groups were compared using the Student t-test.

## RESULTS

In the course of a screening of phytomedicines for their effect on the Nrf2 pathway we tested an aqueous extract of pau d'arco on HepG2 cells that expressed luciferase under the control of eight ARE. The reporter-gene expression was strongly induced by dilutions of pau d'arco extract in a concentration-dependent manner with a maximal activation of ~40-fold and an $EC_{50}$ of 0.7% (v/v) (Fig. 1A). A similar induction of reporter-gene expression was also observed with an aqueous extract obtained from extracting pau d'arco tea (data not shown). Concentrations of both preparations higher than 2% (v/v) decreased the reporter-gene expression, indicating toxicity (data not shown). The induction of luciferase expression by bardoxolone-methyl was synergistic to that by pau d'arco (data not shown). For the subsequent experiments pau d'arco was used at a concentration of 0.5% (v/v). The extract increased the endogenous expression of NQO1 approximately 3-fold, but had only a minor, not significant effect

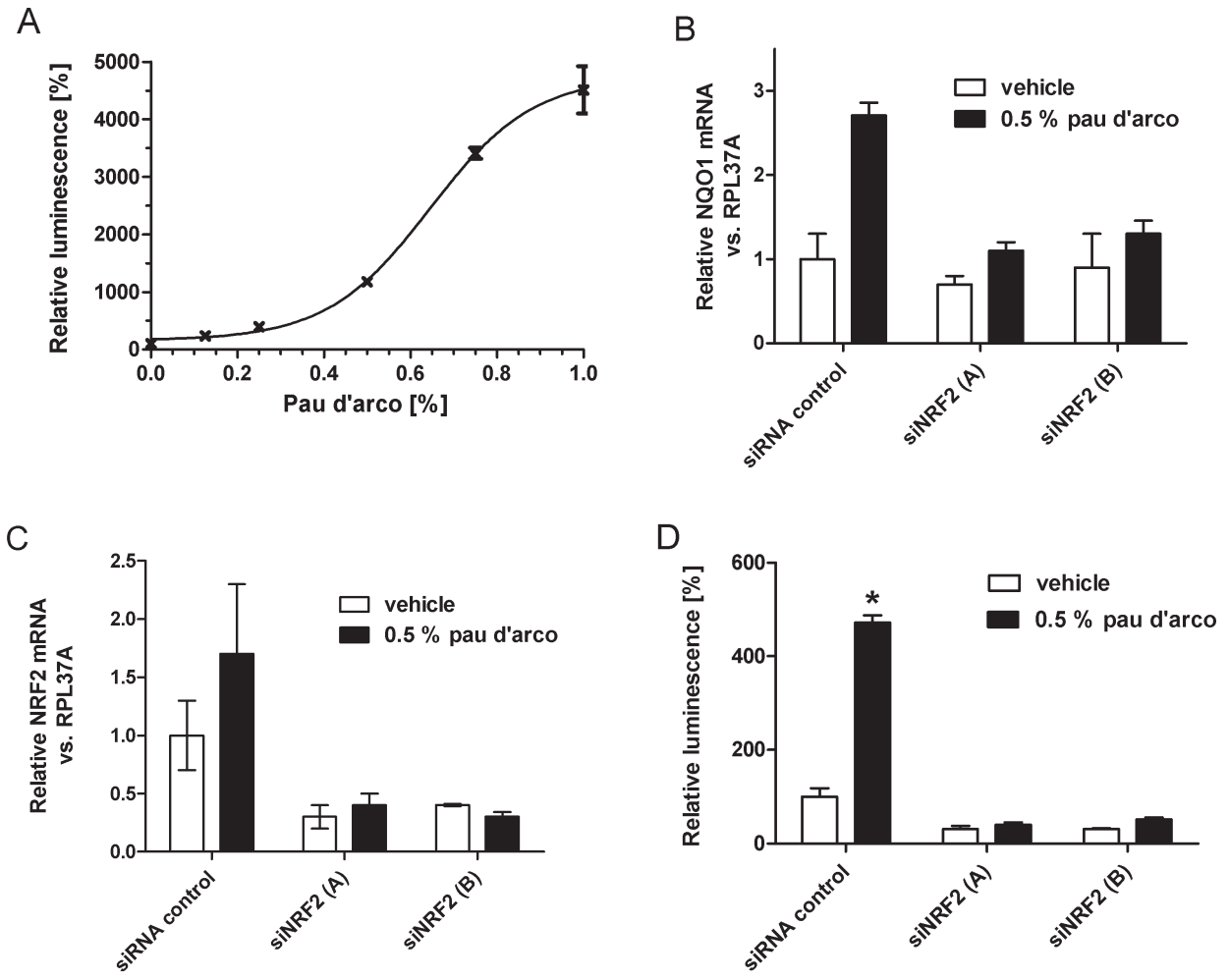


**Fig. 1.** Activation of Nrf2-dependent gene expression by pau d'arco. A) Dose-dependent induction of ARE-driven luciferase expression in stable transfected HepG2 cells by pau d'arco: cells were seeded in 96-well plates and incubated overnight with the indicated concentration of the aqueous extract of pau d'arco. Subsequently, luciferase gene expression was determined. Data are mean ± S.E.M. (n = 3) and the basal luciferase activity in the absence of pau d'arco was set 100%. B,C) Induction of endogenous NQO1 expression by pau d'arco requires Nrf2: HepG2 cells were transfected by either an siRNA control oligonucleotide or two different oligonucleotides specific for Nrf2. Cells were incubated in the presence or absence of pau d'arco. The expression of NQO1, Nrf2 and RPL37A as internal standard were determined by RT-qPCR. Data are presented as mean ± S.E.M. (n = 3) relative to the expression of vehicle-treated siRNA control, which was set as 1. D) Nrf2 mediates induction of ARE-driven luciferase gene expression by pau d'arco: stable transfected HepG2 cells were transfected either with a control oligonucleotide or two different Nrf2-specific probes. Cells were incubated in the presence or absence of pau d'arco overnight, before luciferase expression was determined. Data are presented as mean ± S.E.M (n = 3) relative to the basal luciferase activity of the non-silencing control, which was set 100%; *$p < 0.05$ vs. control.

on Nrf2 mRNA (Fig. 1B, C). The transfection of cells with two different siRNAs for Nrf2 lowered endogenous Nrf2 mRNA levels by approximately 60% compared to a control oligonucleotide (Fig. 1C) and impaired both the increase of NQO1 mRNA (Fig. 1B) and the induction of the reporter gene by the pau d'arco extract (Fig. 1D). In conclusion, pau d'arco activated the expression of both the reporter gene and endogenous NQO1 in an Nrf2-dependent manner.

To analyse its composition, the aqueous extract of pau

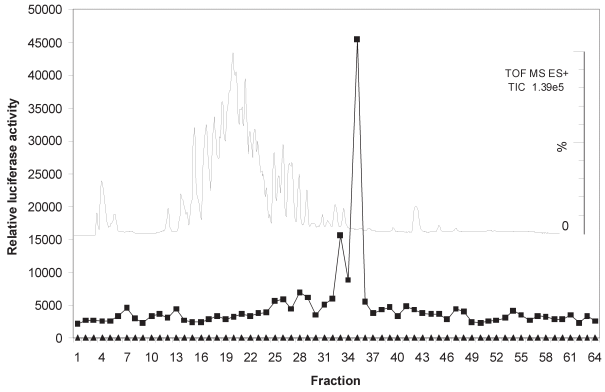


**Fig. 2.** HPLC-MS analysis of the aqueous pau d'arco extract. The pau d'arco extract was separated by HPLC with MS detection (insert) and the effect of the fractions was tested on Nrf2-dependent induction of luciferase gene expression measured by an increase of luminescence. The main activities were found in the fractions 33-35.

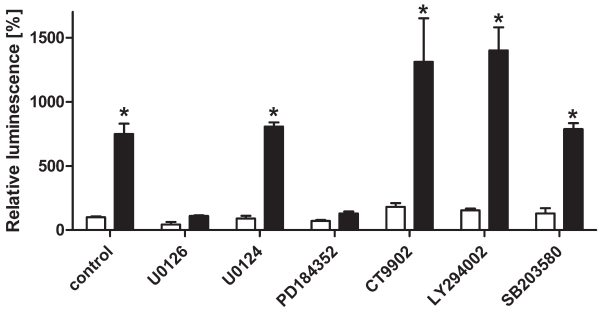


**Fig. 3.** Influence of protein kinases on regulation of Nrf2-mediated luciferase expression by pau d'arco. Stable transfected cells were incubated in the presence (filled bars) or absence (empty bars) of 0.5% pau d'arco and the MEK-inhibitors U0126 (10 µM) and PD184352 (2 µM), with U0124 (10 µM), an U0126-related, but inactive compound, the GSK-inhibitor CT9902 (5 µM), the PI3K-inhibitor LY294002 (100 µM) and the p38 inhibitor SB203580 (5 µM). After incubation overnight, inhibition of luciferase expression was determined. Data are presented as mean ± S.E.M (n = 3) relative to the basal luciferase activity of the vehicle-treated control in the absence of pau d'arco, which was set 100%; *$p < 0.05$ vs. control.

d'arco was fractionated by HPLC with MS detection. The majority of molecules were eluted within the fractions 13-28 as shown by the MS total ion current chromatogram (Fig. 2, insert). All fractions were tested for their ability to induce reporter-gene expression in HepG2 cells. The highest activities were detected in fractions 33 to 35 (Fig. 2). UHPLC-MS analyses of these fractions revealed a complex mixture of various compounds including benzene trioles and benzene tetraoles; several of them were described in the literature as constituents of pau d'arco. However, within the detection limit of 1 µM no quinoids, such as lapachol, and flavonoids, such as quercetin were found within these fractions. Synthetic lapachol and quercetin stimulated the reporter-gene expression by 50% at concentrations of 20 µM and 10 µM, respectively. Their effects on luciferase induction were additive, but not synergistic (data not shown). These data suggest that the activation of Nrf2 by the aqueous extract was not due to the pharmacological lead compounds lapachol and quercetin.

In order to characterize the molecular mechanisms for the activation of Nrf2-dependent gene expression by pau d'arco, selective inhibitors of protein kinases (Bain *et al.*, 2007) were applied that have been linked to the regulation of Nrf2 (Copple, 2012). The induction of the luciferase reporter gene by pau d'arco was blocked by two structurally unrelated inhibitors of mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK), PD184352 and U0126, but not by U0124, an inactive compound structurally-related to U0126 (Fig. 3). Inhibitors of GSK3, p38 MAPK and PI3K did not block the induction of the reporter gene, indicating that these enzymes are not required for the stimulation of Nrf2-regulated gene expression by the plant extract. The inhibitors of MEK also blocked the upregulation of endogenous NQO1 expression by pau d'arco (Fig. 4). Pau d'arco induced phosphorylation of extracellular-signal-regulated kinase (ERK) 1/2, a downstream event of MEK signalling (Fig. 5). This data indicates that the activation of the MEK/ERK pathway is critical for the induction of Nrf2-target genes by pau d'arco.

In order to further characterize the role of MEK/ERK signalling in the stimulation of gene expression by pau d'arco, we studied whether this pathway is required for Nrf2 translocation. Therefore, we performed a DNA-binding ELISA with nuclear extracts prepared from cells that were incubated in the presence and absence of pau d'arco and the MEK inhibitors. By binding to an oligonucleotide with the sequence of the Nrf2-binding site, activated Nrf2 was detected in nuclear extracts derived from cells preincubated with pau d'arco (Fig. 6A). Extracts isolated from vehicle-treated cells did not exhibit activated Nrf2, thereby indicating nuclear translocation of Nrf2 in response to pau d'arco. However, addition of the MEK inhibitors U0126 or PD184352, respectively, did not significantly reduce the nuclear translocation. Next we want-

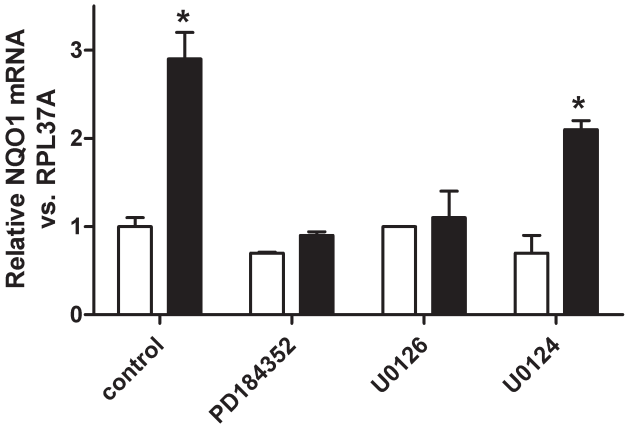


**Fig. 4.** MEK-inhibition reduces the induction of endogenous NQO1 expression by pau d'arco. Cells were incubated for 6 hr with the MEK-inhibitors PD184352 (2 μM), U0126 (10 μM), or with the U0126-related, but inactive compound U0124 (10 μM) in the presence (filled bars) or absence (empty bars) of 0.5% pau d'arco. The expression of NQO1 and RPL37A as internal standard were determined by RT-qPCR. Data are presented as mean ± S.E.M. (n = 3) relative to the expression of vehicle-treated control cells, which was set as 1; *p < 0.05 vs. control.

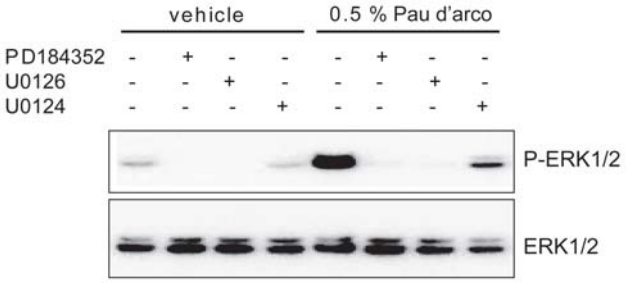


**Fig. 5.** Pau d'arco induces ERK1/2 phosphorylation. HepG2 cells were incubated for 20 min with the MEK-inhibitors PD184352 and U0126, as well as with U0124, an U0126-related, but inactive compound in the presence of absence of pau d'arco. Cell lysates were separated by SDS/PAGE and transferred onto nylon membranes. Western blots were performed using an antibody specific for phosphorylated ERK1/2. As loading control, blots were subsequently incubated with anti-ERK1/2 antibody.

ed to visualize nuclear localization of Nrf2 directly. As we were unable to detect endogenous Nrf2 levels by immunofluorescence studies, we transiently transfected cells with a plasmid expressing HA-tagged Nrf2. We failed to detect Nrf2 expression using an anti-HA antibody (data not shown), indicating that the HA-tag of the recombinant protein was not accessible to the antibody. However, cellular staining of transfected cells was achieved by an anti-Nrf2 antibody. In relation to vehicle-treated cells the incubation with pau d'arco increased the nuclear localization of Nrf2, as demonstrated by a nuclear counterstain (Fig. 6B). The MEK inhibitors did not significantly prevent the nuclear localization of overexpressed Nrf2. Overall, these data indicate that MEK signalling is not required for the nuclear translocation of Nrf2 in response to pau d'arco, although it is required for the induction of Nrf2-target genes by the plant extract (Fig. 4).

Electrophilic compounds activate Nrf2 by reacting with cysteine residues of Keap1. This can be blocked by increasing the extra- and intracellular concentration of free cysteine residues (Hur *et al.*, 2010). In line with that, the incubation in the presence of N-acetyl cysteine (NAC) partly blocked the induction of endogenous NQO1 expression by the electrophilic compounds tert-butylhydroquinone (tBHQ) and sulforaphane (SFN) (Fig. 7). However, NAC had no significant effect on the regulation of NQO1 by pau d'arco. Thus, these data indicate that pau d'arco induces nuclear localization of Nrf2 and gene expression by the MEK-ERK pathway, but this does not depend on a direct modification of cysteine residues of Keap1 by a highly reactive component of the extract.

Next we assessed if pau d'arco is able to stimulate Nrf2 *in vivo* by analysing the expression of the prototypical Nrf2-target genes HMOX1 and NQO1 in mice. Pau d'arco was administered twice at a dose of 5,000 mg/kg. In agreement with our *in vitro* results, pau d'arco acutely increased the expression of HMOX1 and NQO1 in the intestine in particular within the duodenum (Fig. 8). We found no induction of gene expression in liver, kidney, blood and lung (data not shown).

## DISCUSSION

Pau d'arco is used in South American medicine for the treatment of a broad range of diseases (Gómez Castellanos *et al.*, 2009). However, its molecular mode of action is not well characterized. The present study demonstrates an activation of the cytoprotective Nrf2 pathway by aqueous extracts prepared from two different sources of pau d'arco. Pau d'arco activated MEK/ERK signalling and this was required for its effect on Nrf2 target genes. A regulation of Nrf2 by MEK/ERK has also been described for flavonoids, epigallocatechin gallate and oleanolic acid (Yang *et al.*, 2011; Na *et al.*, 2008; Feng *et al.*, 2011). In contrast to the induction of Nrf2 target genes, the nucle-

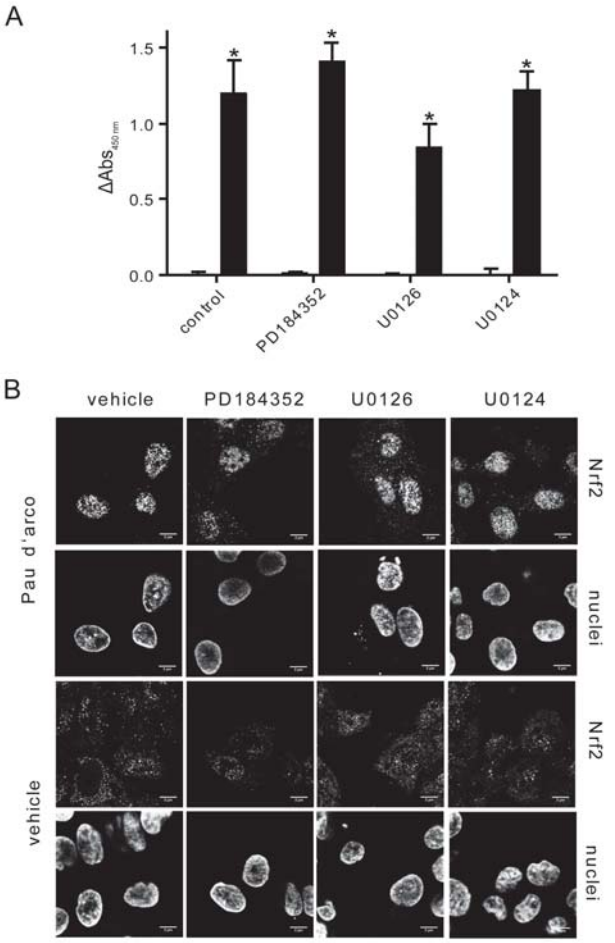


**Fig. 6.** Pau d'arco promotes nuclear localization of Nrf2 independently of MEK activation. A) Nrf2 binding assay: HepG2 cells were incubated for 6 hr with the MEK-inhibitors PD184352, U0126, or with U0124, an U0126-related, but inactive compound in the presence (filled bars) or absence of 0.5% pau d'arco (empty bars). Nuclear extracts were prepared and binding assays towards an immobilized oligonucleotide with the sequence of the Nrf2-binding site were performed as described in Materials and Methods. Data presented are the differences of the absorbance between binding reactions containing competing mutated oligonucleotide and the wildtype oligonucleotide ($\Delta Abs_{450nm}$) and are presented as mean ± S.E.M. (n = 3). *p < 0.05 vs. preincubation with vehicle. B) Confocal laser scanning microscopy: HepG2 cells were transiently transfected with a plasmid expressing HA-Nrf2. After 72 hr cells were incubated for 2 hr in the absence or presence of pau d'arco and the MEK-inhibitors PD184352 and U0126, as well as U0124, an U0126-related, but inactive compound. Shown are representative immunofluorescences obtained with an anti-Nrf2 antibody and the corresponding nucleic staining; scale bar = 2 µm.

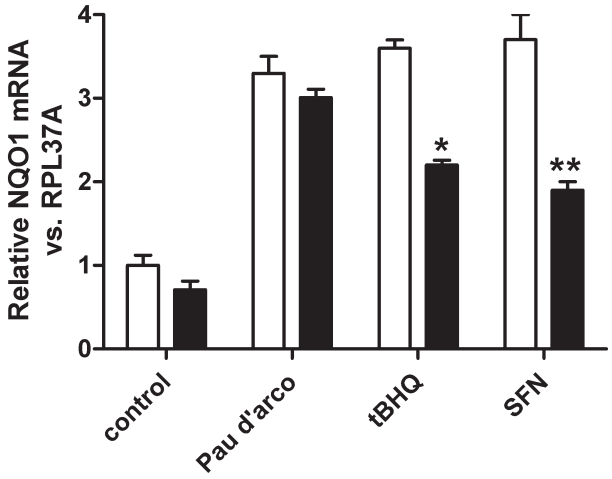


**Fig. 7.** NAC blocks the induction of endogenous NQO1 expression by electrophilic Nrf2-inducers sulforaphane and tBHQ, but not by pau d'arco. Cells were incubated for 6 hr with our without 0.5% pau d'arco, 10 µM sulforaphane (SFN), 10 µM tBHQ in the presence (filled bars) or absence (empty bars) of 1 mM NAC. The expression of NQO1 and RPL37A as internal standard were determined by RT-qPCR. Data are mean ± S.E.M. (n = 3) and shown as relative to the expression of control cells, which was set as 1. *p < 0.05 vs. tBHQ alone, **p < 0.01 vs. SFN alone.

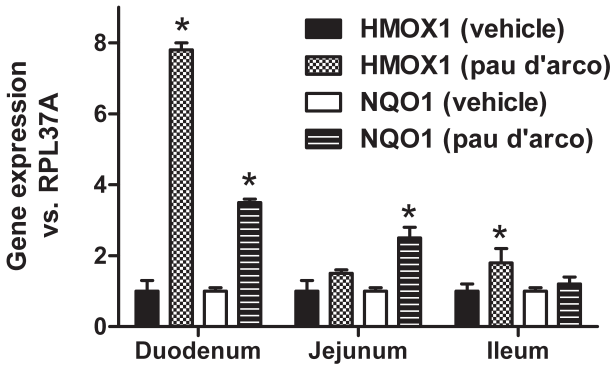


**Fig. 8.** Induction of Nrf2-target gene expression in the intestine by pau d'arco. Mice were treated twice with pau d'arco (5,000 mg/kg) or vehicle. The animals were killed 5 hr after the second application and the tissues removed for RNA isolation. Gene expression analysis was performed by RT-qPCR. Results are expressed as NQO1 and HMOX1 gene expression, respectively relative to the internal standard RPL37A. Data are presented as mean ± S.E.M (n = 4) relative to the expression in vehicle-treated animals, which was set as 1. *p < 0.05 vs. respective vehicle-treated animals.

ar translocation of Nrf2 in response to pau d'arco did not depend on MEK signalling, as shown by both binding assays and immunofluorescence studies. Therefore, we propose that the activation of the MEK/ERK pathway is not required for the activation of Nrf2 by pau d'arco, but acts via a secondary mechanism such as the regulation of a co-activator on the regulation of Nrf2-target genes by pau d'arco. This is supported by the previous observation that the direct phosphorylation of Nrf2 by ERK has only a minor effect on Nrf2 activity (Copple, 2012; Sun *et al.*, 2009). Interestingly, we observed a more than additive effect of pau d'arco and bardoxolone methyl, which is known to modify Keap1. As we did not observe activation of the MEK pathway by bardoxolone methyl, we speculate that the activation of MEK by pau d'arco might have a synergistic effect on Nrf2 activation by bardoxolone methyl.

The failure of NAC to impair gene regulation by pau d'arco excludes that the regulation of Nrf2 is caused by a highly electrophilic ingredient of the extract. Interestingly, the chromatographic separation of the extract showed that the regulation of Nrf2 was not caused by lapachol and quercetin. These compounds have been associated with pharmacological actions of pau d'arco (Gómez Castellanos *et al.*, 2009). Instead we found within the active fractions a complex mixture of compounds including benzene derivates. It is unclear if and which of these compound classes are able to activate Nrf2. This must be addressed by testing chemically pure reference compounds obtained by de novo synthesis that was beyond the scope of the present paper. The isolated compound classes have pro-oxidative properties, and reactive oxygen species regulate both MEK and Keap1 (Copple, 2012; Rahman *et al.*, 2006; Runchel *et al.*, 2011; Owuor and Kong, 2002). We speculate that the extract generates a low-grade oxidative stress that was not blocked by NAC and synergistically contributes to the activation of both MEK and Nrf2. The identification of the active ingredient(s) will be required to further elucidate the molecular mechanism(s) for the activation of MEK and Nrf2 in response to pau d'arco.

In order to assess if pau d'arco is able to activate Nrf2 *in vivo* and to determine the target tissues of this regulation, we analysed the expression of Nrf2 target genes HMOX1 and NQO1 after oral dosing in mice. This readout has been applied previously for the assessment of the pharmacokinetics of Nrf2 activators (Yates *et al.*, 2007; Cornblatt *et al.*, 2007). Pau d'arco stimulated the Nrf2 target gene expression only in the upper part of the intestine. Due to a limited availability of animal tissue we were not able to study if MEK/ERK-activity was activated in parallel. The failure of pau d'arco to stimulate Nrf2-target gene expression in blood cells, kidney, liver and lung, indicates that the active component has a poor pharmacokinetic profile and could either be rapidly inactivated or eliminated. Furthermore, in our experiments we used an aqueous extract. However, chronic application of pau d'arco might increase the systemic availability of the active ingredient and the activation of Nrf2 in additional tissues. Importantly, the activation of Nrf2 in the intestine was shown to be protective in models of colitis (Khor *et al.*, 2006; Kim *et al.*, 2013; Wagner *et al.*, 2013). We have described here that pau d'arco activates Nrf2-signalling in the intestine. With that, the Nrf2 pathway could mediate the beneficial gastro-intestinal effects of this phytomedicine (Gómez Castellanos *et al.*, 2009).

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