APPLICATION OF THE *HMOX1* PROMOTER/ENHANCER ASSAY FOR ROUTINE DETERMINATION OF CELLULAR STRESS INDUCED BY CIGARETTE SMOKE

Constanze Knörr-Wittmann*, Stephan Gebel, Thomas Müller; PHILIP MORRIS Research Laboratories GmbH, Cologne, Germany; *e-mail: Constanze.Knoerr@pmintl.com

Introduction

Cigarette smoke (CS) harbors a strong oxidative stress potential, which broadly impacts exposed cells. A special feature of the CS-provoked cellular stress response is the strong transcriptional up-regulation of phase II and antioxidant-related genes, among which the expression of the gene encoding heme oxygenase-1 (*hmox1*) is most prominent. This has been seen in *in vitro* and *in vivo* cDNA microarray studies [1, 2], suggesting that HO-1 plays a central role in the cellular defense mechanisms against oxidative stress. HO-1 catalyzes the rate-limiting reaction in the catabolism of heme, yielding products with both antioxidant and cytoprotective activities. Based on these characteristics, as well as on the strong transcriptional inducibility under various unrelated stressing conditions, the expression of *hmox1* is considered a reliable marker of cellular (oxidative) stress.

We previously presented first results of the *hmox1* promoter/enhancer assay [3], demonstrating that a *hmox1* promoter/enhancer reporter (*luciferase*) gene construct stably transfected into NIH3T3 cells is responsive to cigarette smoke fractions and single compounds at subcytotoxic concentrations. Here we present an optimized protocol for a higher (96-well format *vs* single petri dish) and faster (1 day *vs* 3 days) sample through-put.

Objective

Optimize the *hmox1* promoter/enhancer assay for a high through-put routine determination of cellular (oxidative) stress.

Materials and Methods

The transgenic cell line NIH3T3/pHO15luc was created by transfection of NIH3T3 cells with the plasmids pHO15luc (kindly provided by Dr. J. Alam) containing the 15 kb promoter/enhancer region of the murine *hmox1* gene fused to the firefly luciferase gene (Fig. 1).

NIH3T3/pHO15luc cells in culture were kept under standard conditions with 400 mg/ml zeocin. For testing various concentrations of CS fractions or single compounds in quadruplicate, cells were seeded in black 96-well plates, starved (24 hours, 0.5% FCS) and then exposed in DMEM/0.5% FCS. After 7 hours WST-1 Cell Proliferation Reagent (Roche, Germany) was added to each well and cell proliferation/viability was determined. After a washing step, luciferase activity was measured directly in each well with the 'Luciferase Reporter Gene Assay'-Kit (Roche, Germany). Chemiluminescence was determined and quantified by the luminometer "Fluostar Optima BMG" (BMG Labtechnologies, Germany). Results from the WST-1 Assay were used for normalization of each well on the basis of viable cell number. Luciferase activity was expressed as 'Fold Induction' of treated to control samples. SD (standard deviation) was determined for 'Fold Induction'. Wells showing cytotoxic effects, as detected by the WST-1 assay, were not used for analysis.

Cigarette Smoke Fractions: Smoke-bubbled PBS (sbPBS) was prepared by bubbling 30 puffs of mainstream CS from the Standard Reference Cigarette 2R1 through 18 ml PBS-Dulbecco. Gas/vapor phase (GVP) was prepared in the same way except that the smoke was passed through a glass fiber filter (44 mm diameter, Borgwaldt Technik GmbH, Germany). Total particulate matter (TPM) from three cigarettes (2R1 or 2R4F) was collected on one glass fiber filter (44 mm diameter), determined gravimetrically, and then extracted with 10 ml dimethyl sulfoxide (DMSO).

Results

1. *hmox1* Promoter/Enhancer Region

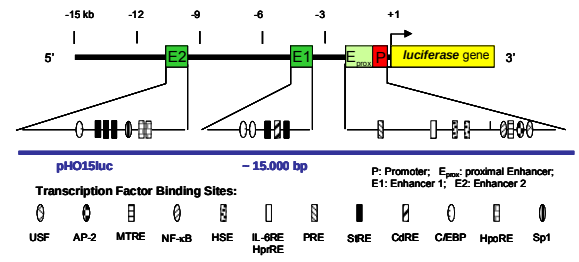


Fig. 1
The 15kb *hmox1* promoter/enhancer region fused to the firefly luciferase gene was used to generate the transgenic cell line NIH3T3/pHO15luc.

2. Biological Significance

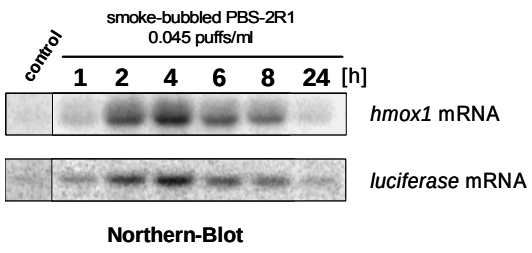


Fig. 2
CS-dependent endogenous *hmox1* and ectopic luciferase gene expression in NIH3T3/pHO15luc cells are regulated in a similar way.

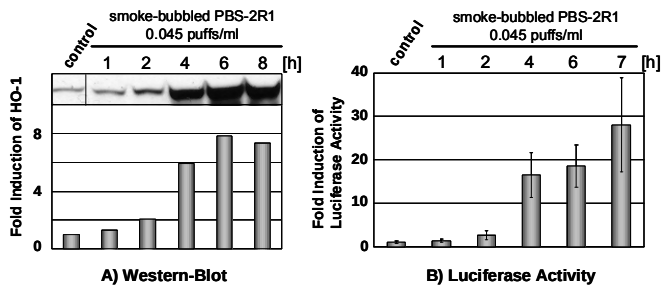
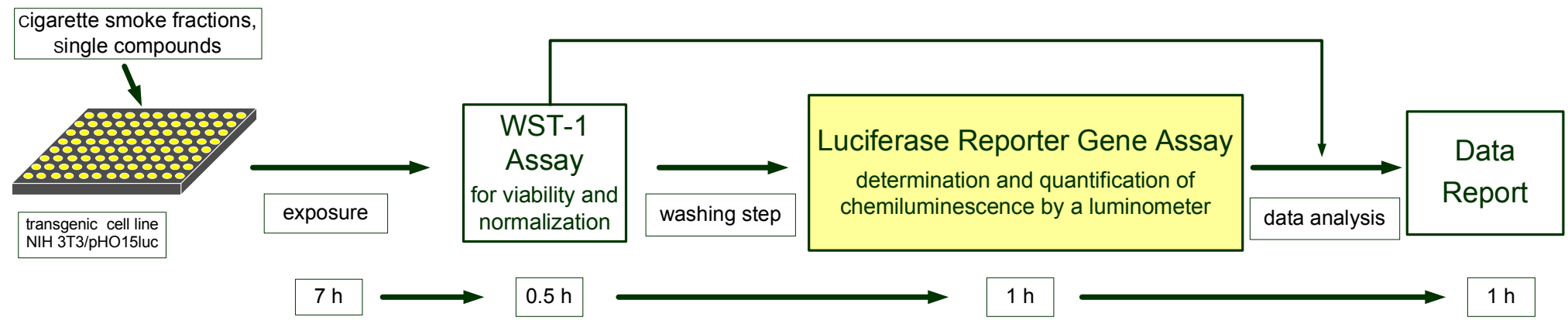


Fig. 3
Induction of endogenous HO-1 protein expression (A) correlates with ectopic luciferase activity (B).

3. *hmox1* Promoter/Enhancer Assay Design



4. First Application

$CdCl_2$

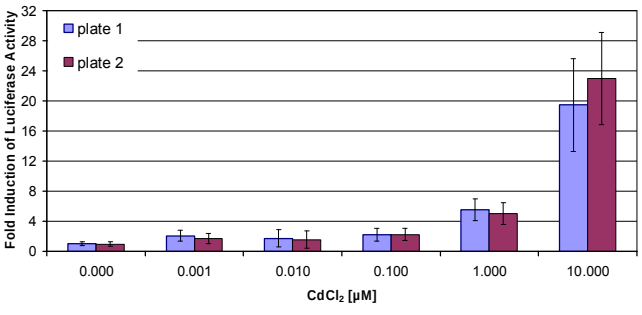


Fig. 4
Concentration-dependent activation of luciferase activity by $CdCl_2$ (used as positive control); two independent experiments on one day.

TPM (total particulate matter)

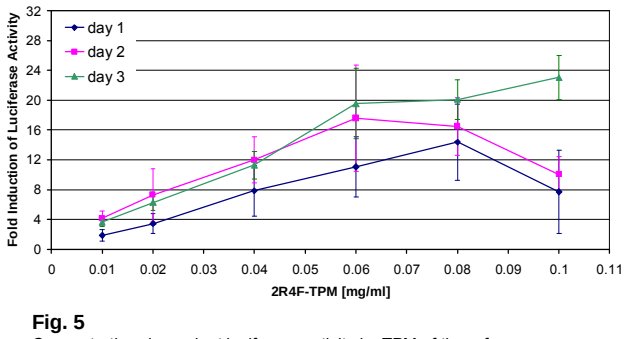


Fig. 5
Concentration-dependent luciferase activity by TPM of the reference cigarette 2R4F; three independent experiments on three days.

GVP (gas/vapor phase) and TPM

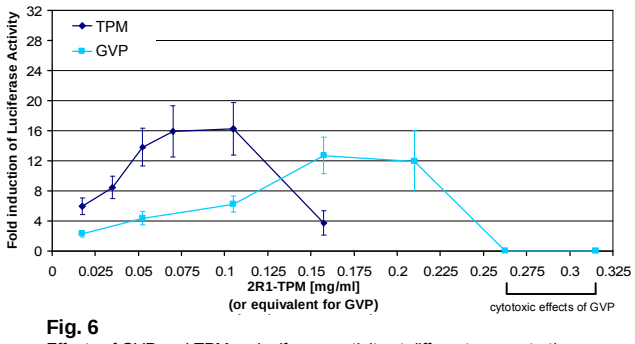


Fig. 6
Effects of GVP and TPM on luciferase activity at different concentrations. Equal GVP and TPM concentrations are calculated on a puff basis.

Summary

The *hmox1* promoter/enhancer *in vitro* assay has been optimized for routine determination of cellular stress using cigarette smoke fractions and single compounds.

- Luciferase expression reflects wild-type induction of *hmox1*.
- 23 test samples in quadruplicate can be analyzed and compared on one plate.
- The assay can be performed in a 96-well format within one day.
- Intraday and interday variability were very low indicating that the assay would be useful for measuring cellular (oxidative) stress.

Literature

[1] Bosio, A., Knörr, C., Janssen, U., Gebel, S., Haussmann, H.-J., Müller, T. (2002) Carcinogenesis **23**, 741-748.

[2] Gebel, S., Gerstmayer, B., Bosio, A., Haussmann, H.-J., Van Miert, E., Müller, T. (2004) Carcinogenesis **25**, 169-178.

[3] Knörr, C., Gebel, S., Müller, T., (2004) Toxicol. Sci. **78** (Suppl. 1), 308-308.

Acknowledgement

We thank J. Alam (Ochsner Clinic Foundation, New Orleans) for providing a sample of plasmid DNA pHO15luc and G. Stellbrink for skilfull technical assistance.

EUROTOX, Cracow, 11 to 14 September 2005

Lebrun, S.; Stinn, W.; Weiler, H.; Kuhl, P.; Friedrichs, B.; Urban, H.-J.; von Holt, K.; Wallerrath, T.; Schleef, R., "Initial data on the effects of long-term cigarette mainstream smoke exposure and high fat diet on Apolipoprotein E-deficient mice". Toxicology Letters 158(S1): 74; 2005. Poster presented at the 42nd Congress of the European Societies of Toxicology, Cracow, Poland, September 11-14, 2005 and at the International Society on Thrombosis and Haemostasis XXth Congress, Sydney, Australia, August 6-12, 2005.