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A high-throughput reporter gene assay to prove the ability of natural compounds to modulate glutathione peroxidase, superoxide dismutase and catalase gene promoters in V79 cells

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Abstract
The aim of the study was to establish a 96-well microtiter plate-based reporter gene assay to test the influence of natural compounds on the promoter activities of rat catalase, human glutathione peroxidase and human superoxide dismutase expressed in V79 cells. Luciferase expression vectors with the promoter regions of the genes coding for the three above-mentioned enzymes were constructed and transfected into V79 cells. Thereafter the ability of sodium ascorbate, L-carnitine, catechin, epigallocatechin gallate, genistein, paraquat, quercetin, 12-*O*-tetradecanoylphorbol-13-acetate and Trolox to enhance the promoter activities was evaluated. Genistein, paraquat and quercetin led to a statistically significant increase in the glutathione peroxidase and superoxide dismutase gene promoter activities. None of the compounds tested enhanced the catalase gene promoter activity. The reporter gene assay described in this report is easy to perform, fast and allows one to test a high number of compounds and different concentrations of a single compound at the same time.

Keywords: *Catalase, glutathione peroxidise, superoxide dismutase, reporter gene assay, natural compounds*

Introduction

A number of metabolic processes in normal cells lead to the formation of reactive oxygen species (ROS). These include the superoxide radical, the hydroxyl radical and hydrogen peroxide. High intracellular levels of ROS may not only damage proteins and lipids but also alter DNA, thereby supporting cancer development. Cells are able to protect proteins, lipids and DNA from the deleterious effects of ROS by a number of antioxidative mechanisms. These mechanisms include intracellular reductants such as glutathione and some vitamins and enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT).

The first step in limiting enzymatically ROS-induced cellular damage is the conversion of the superoxide radical to hydrogen peroxide by SOD [1].

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If SOD is unable to substantially decrease the intracellular superoxide radical levels, these can react with nitric oxide and generate peroxynitrite, which in turn can nitrate tyrosine residues in cellular proteins, thereby altering the functions of these proteins [2,3]. In the case that SOD successfully converts the superoxide radical to hydrogen peroxide, hydrogen peroxide may be further toxified in the presence of ferrous ions by converting it to the hydroxyl radical via the Fenton reaction [4]. Since the hydroxyl radical is the most reactive ROS, capable of reacting with virtually all vital cellular macromolecules, hydrogen peroxide must be detoxified as quickly as possible. This is accomplished by GPx as well as CAT by converting hydrogen peroxide to water and oxygen. Hence, it is the coordinated interplay of SOD, GPx and CAT that makes it possible to limit oxidative stress in cells.

Copper/zinc SOD (SOD1) is located in the cytoplasm, while manganese SOD (SOD2) is present in mitochondria. All members of the GPx family reduce hydroperoxides by means of glutathione, whereby the individual members of this enzyme family accomplish different functions: GPx1 is an antioxidant device, whereas GPx4 (phospholipid hydroperoxide GPx) is essential for male fertility and embryonic development [5]. GPx1 is localized in the cytoplasm and in mitochondria, i.e. in the immediate vicinity of SOD1 (cytoplasm) and SOD2 (mitochondria). CAT is specifically located in peroxisomes, in which it is needed to decompose hydrogen peroxide, mainly being generated during the β-oxidation of fatty acids, in enzymatic reactions catalysed by flavin oxidases as well as during the disproportionation of superoxide radicals [6]. Thus, the distribution of SOD, GPx and CAT within eukaryotic cells also supports the view that these enzymes play an important role as a first line of defence against oxidative stress.

Since ROS have been implicated in a variety of pathological conditions in humans such as cancer, inflammation, cardiovascular diseases and diabetes, it has been speculated that the endogenous enzymatic and non-enzymatic defence mechanisms present in the human body might not be sufficient to avoid oxidative stress and that dietary antioxidants could help to enhance the antioxidant capacity in humans [7,8]. In order to do so, one could make use of compounds with a direct radical scavenging activity or compounds with the ability to enhance the expression of enzymes with antioxidant activity [9].

From a practical point of view an *in vitro* assay to test the ability of natural and synthetic compounds to induce enzymes with antioxidant activity would have a number of advantages over *in vivo* studies. On the one hand, it would be possible to avoid the use of experimental animals. On the other hand, it would enable the testing of a high number of compounds in a short period of time. In a first step in this direction Toyokuni et al. [10] constructed luciferase expression vectors with the promoter regions of the genes coding for SOD1, SOD2, GPx and CAT and transfected them into COS-7 cells (non-neoplastic renal tubular cells derived from an African green monkey) cultured in 12-well plates. With this experimental system Toyokuni et al. [10] showed that certain Mauritian plant extracts are indeed able to enhance the promoter activities of SOD1 and GPx1.

The aim of the present study was to develop a high-throughput assay to screen a high number of natural compounds within a short period of time. Therefore, in a first step the transfection of the luciferase expression vectors with the promoter regions of the genes coding for human SOD1, human GPx1 and rat CAT into cells cultured in 96-well microtiter plates was established. In the present study the easy to handle V79 cells, Chinese hamster lung fibroblasts routinely included in a number of *in vitro* test systems to evaluate the toxicity of chemicals, were used instead of COS-7 cells. In a second step the ability of sodium ascorbate (AS), L-carnitine (LC), catechin (CA), epigallocatechin gallate (EGCG), genistein (GN), paraquat (PQ), quercetin (QU), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and Trolox (TX) to enhance the above-mentioned promoter activities in V79 cells was evaluated. Hydrogen peroxide (HP) was only tested in V79 cells expressing the rat CAT promoter. From HP, PQ and TPA it is known that they act as inducers of at least one out of three of the antioxidant enzymes analysed in the present study, whereas CA, EGCG, GN and QU are antioxidative acting food flavonoids capable of regulating the activities of antioxidative enzymes (the corresponding studies to each compound are cited in the Discussion section). In the case of the well known antioxidants AS, LC and TX, no such information was available in the scientific literature so they were analysed herein.

Materials and methods

Chemicals

AS, LC, CA, EGCG, PQ, QU, TPA and TX were purchased from Sigma-Aldrich Chemie (Munich, Germany), GN from AppliChem (Darmstadt, Germany) and HP from Carl Roth (Karlsruhe, Germany). The Alamar Blue® stock solution for the cytotoxicity assays was obtained from Morphosys AbD (Düsseldorf, Germany).

Plasmids

pGL4.83, which includes the *Renilla* luciferase gene sequence as well as the ampicillin and the puromycin resistance genes, and phRL-TK, which contains the thymidine kinase promoter of the *Herpes simplex* virus, were purchased from Promega (Mannheim,

Germany). pGL3-SOD, pGL3-GPx and pGL3-CAT, which encode the human SOD1 promoter (−1499 to +27; the transcription start site is defined as +1), the human GPx1 promoter (−1389 to +565) and the rat CAT promoter (−3307 to +2), respectively, as well as firefly luciferase, were kindly provided by S. Toyokuni (Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan); their construction was previously described by Toyokuni et al. [10].

In order to clone the thymidine kinase promoter of the *Herpes simplex* virus into pGL4.83, pGL4.83 and phRL-TK were digested with *Bgl*II and *Hind*III (Promega) as follows: 3 µl (3 µg) DNA, 1 µl (10 U) *Bgl*II, 1 µl (10 U) *Hind*III, 2 µl NEBuffer 2 (10X) (New England Biolabs, Ipswich, MA) and 13 µl dist. water were first incubated at 37°C for 2 h and then at 65°C for 15 min. The fragments were separated in a 1% agarose gel and extracted from the gel by using the MicroElute™ Gel Extraction Kit from Omega Bio-Tek (Doraville, GA). The plasmid pGL4-TK was obtained by incubating 100 ng of the digested pGL4.83, 33 ng of the thymidine kinase promoter insert, 3 U T4-DNA-ligase (Promega), 5 µl 2X Rapid Ligation Buffer (Promega) and 0.75 µl distilled water for 30 min at room temperature.

Thereafter, *Escherichia coli* DH5α (in the case of pGL3-SOD, pGL3-GPx and pGL3-CAT) and *Escherichia coli* TOP10 (in the case of pGL4-TK) from Invitrogen (Karlsruhe, Germany) were used to amplify the plasmids. Briefly, 1 ng DNA was added to 50 µl of the *Escherichia coli* DH5α suspension and electroporated at 2500 V for a few seconds. Immediately afterwards 950 µl SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$ and 20 mM glucose) were added. The bacterial suspension was transferred to a 15-ml test tube and incubated at 37°C for 1 h. In the case of *Escherichia coli* TOP10 100 µl of the bacterial suspension were mixed with the ligation mix (in total 133 ng DNA) and incubated for 30 min on ice, for 30 s at 42°C and for 2 min again on ice. Subsequently, 250 µl SOC medium were added and the bacterial suspension was incubated at 37°C for 45 min. The transformed bacteria were seeded on LB plates (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar) supplemented with ampicillin (100 µg/ml). After incubating the plates at 37°C overnight single colonies were propagated in LB medium. Plasmid DNA was isolated from the bacteria by using the HiSpeed® Plasmid Midi Kit from Qiagen (Hilden, Germany) and the base sequence of each plasmid was checked by direct DNA sequencing.

Cell culture

V79 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany and grown in Dulbecco's modified Eagle's medium (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin (Biochrom).

Transfection of plasmids into V79 cells and incubation of the transfected cells with the test compounds

Eight thousand V79 cells in 200 µl cell culture medium were seeded in each well of a 96-well microtiter plate (TPP, Trasadingen, Switzerland) and cultured at 37°C for 24 h. The transfection mix per well consisted of 1 µg pGL3-SOD, pGL3-GPx or pGL3-CAT, 100 ng pGL4-TK, 15 µl distilled water, 5 µl 1 M $CaCl_2$ and 20 µl phosphate buffer (50 mM HEPES, 1.26 mM Na_2HPO_4, 140 mM NaCl). After carefully dropping 40 µl of the transfection mix in each well the 96-well microtiter plate was incubated at 37°C for 5 h. Thereafter, the cell supernatant in each well was replaced by 50 µl of a 15% glycerol solution (18.9 g glycerol and 0.877 g NaCl in 100 ml distilled water) in an isotonic HEPES buffer (0.238 g HEPES in 80 ml distilled water, pH 7.5). After incubating the cells for 3 min at room temperature these were washed twice with 100 µl serum-free cell culture medium and 200 µl of fresh cell culture medium with the test compounds were added to each well for 24 h.

Cell lysis

At the end of the 24-h incubation period the cell monolayer in each well was washed twice with 250 µl phosphate buffered saline (PBS; 137 mM NaCl, 5.36 mM KCl, 1.47 mM KH_2PO_4, 8 mM Na_2HPO_4) and the 96-well plate was then quick-frozen in liquid nitrogen. Fifty microlitres Passive Lysis Buffer (Promega) were added to each well, shaken for 15 min at room temperature and incubated for another 45 min on ice.

Dual-luciferase® reporter gene assay

The *Renilla* and the Firefly luciferase activities were measured in 20 µl cell lysate by using the Dual-Luciferase® Reporter Assay System (Promega) according to the instructions of the manufacturer and the Nunclon™ Δ surface 96-well microtiter plates from Fisher Scientific (Schwerte, Germany). The *Renilla* luciferase activity in each well served as internal transfection control and the measured Firefly luciferase activities were normalized to the corresponding *Renilla* luciferase activity in each well. Each experiment was performed three times and the incubation with each test compound was performed in four different wells per experiment.

Cytotoxicity assay

Eight thousand V79 cells in 200 µl cell culture medium were seeded in each well of a 96-well microtiter plate and cultured at 37°C for 24 h. Then, the cell supernatants were removed and 200 µl cell culture medium containing the test compounds in various different concentrations were added. Twenty-four hours later the medium containing the test compounds was removed, the cells were washed once with PBS and medium with 10% v/v Alamar Blue® was added to each well. Following an incubation period of 3 h fluorescence was measured (excitation wavelength: 530 nm; emission wavelength: 590 nm). The mean fluorescence of four replicate wells for each concentration of a test compound was calculated.

Statistical analysis

The statistical analysis of the results was carried out by performing an analysis of variance (ANOVA) followed by Dunnett's multiple comparison test [11] with the software SPSS version 11 for Windows. The null hypothesis was rejected at the 0.05 level. Results appear as means; error bars represent the standard deviation.

Results

In the present study an *in vitro*-test system to evaluate the ability of test compounds to enhance SOD, GPx1 and CAT promoter activities expressed in V79 cells was established. The plasmids pGL3-SOD, pGL3-GPx or pGL3-CAT, which contain the human SOD1 promoter, the human GPx1 promoter and the rat CAT promoter, respectively, as well as the plasmid pGL4-TK, which includes the *Renilla* luciferase gene sequence, were co-transfected into V79 cells cultured in 96-well microtiter plates by calcium phosphate co-precipitation. After a 5-h incubation with the transfection mix and a 3-min glycerol shock the cells were incubated for 24 h with the test compounds. This temporal sequence was chosen because preliminary experiments had shown that the highest luciferase activities were measured 24 h after transfection. At this time point the firefly and the *Renilla* luciferase activities were measured in the cell lysates, whereby pGL4-TK with the *Renilla* luciferase coding sequence served as the control reporter gene. With the method described in this study a transfection efficiency of 60–80% was routinely reached.

In order to determine the highest concentration of each test compound that did not lead to a statistically significant decrease of cell viability when compared to the solvent control, a cytotoxicity assay was performed. The concentrations that fulfilled this criterium and that were used in the Dual-Luciferase® Reporter Assay System were 500 µM AS, 10 µM TX, 50 µM CA, 1 mM LC, 50 µM EGCG, 10 µM QU and 100 µM PQ. Suzuki et al. [12] had shown that 100 µM GN is able to enhance GPx1 mRNA levels in two human prostate cancer cell lines, while Choi et al. [13] reported that 50 and 100 µM GN led to an increase in SOD and CAT activities in the murine macrophage cell line RAW 264.7. Five micromolar GN proved to be non-cytotoxic for V79 cells, whereas 10 and 50 µM GN led to a 10 and 20% decrease in cell viability, respectively. Based on these results, V79 cells were incubated with 20 µM GN. In the case of TPA the compound was not cytotoxic up to a concentration of 1 µM. However, since TPA had previously been shown to activate the SOD1 gene promoter at a concentration of 0.15 µM [10,14], this concentration was also used in the present study.

The ability of AS, LC, CA, EGCG, GN, PQ, QU, TPA and TX to modulate the human GPx1 promoter activity is shown in Figure 1. From the nine compounds tested only GN, QU and PQ significantly enhanced the GPx1 promoter activity, the increases being 1.6-, 2.0- and 2.5-fold, respectively, when compared to the promoter activity of cells exposed to the solvent DMSO alone. In the case of the human SOD1 promoter activity again only GN, QU and PQ led to a statistically significant activation (Figure 2), the magnitude of the increase in the SOD1 gene

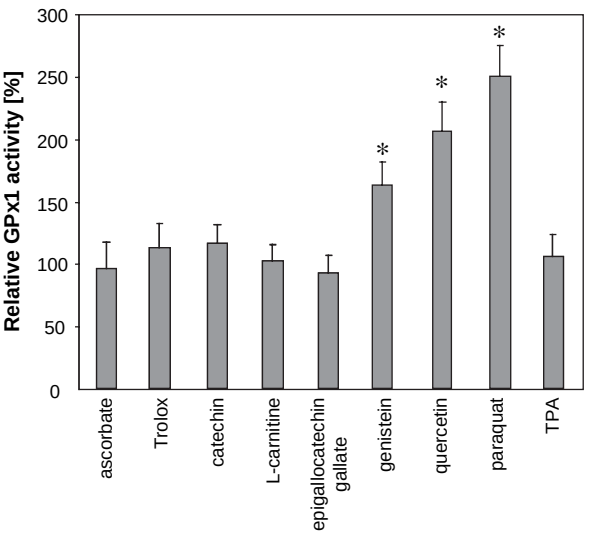

Figure 1. Effect of the test compounds on GPx1 promoter activity. The results are expressed as mean ± SD of the relative GPx1 promoter activities (i.e. the quotient of the GPx1 promoter activity in the presence of the test compound dissolved in DMSO and the GPx1 promoter activity in the presence of DMSO alone × 100). The concentration of each compound in the wells was: 500 µM AS, 10 µM TX, 50 µM CA, 1 mM LC, 50 µM EGCG, 5 µM GN, 10 µM QU, 100 µM PQ and 0.15 µM TPA. The data were obtained from three independent experiments, the incubation with each test compound being performed in four different wells per experiment. The asterisk indicates significantly different from the solvent control value (100%; Dunnett's multiple comparison test, $p < 0.01$).

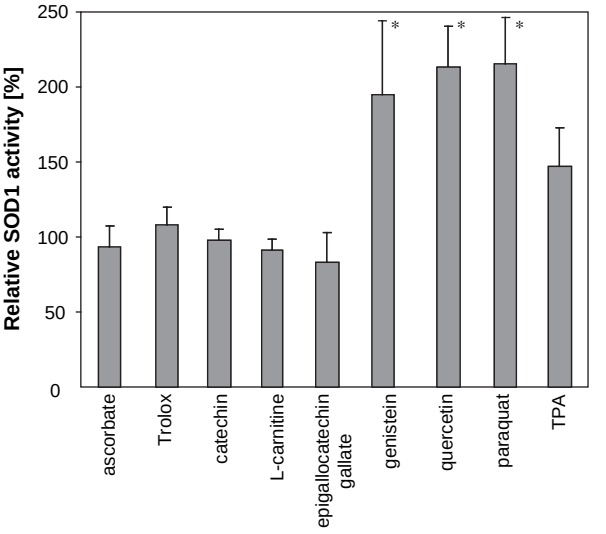

Figure 2. Effect of the test compounds on SOD1 promoter activity. The results are expressed as mean ± SD of the relative SOD1 promoter activities (i.e. the quotient of the SOD1 promoter activity in the presence of the test compound dissolved in DMSO and the SOD1 promoter activity in the presence of DMSO alone × 100). The concentration of each compound in the wells was: 500 μM AS, 10 μM TX, 50 μM CA, 1 mM LC, 50 μM EGCG, 5 μM GN, 10 μM QU, 100 μM PQ and 0.15 μM TPA. The data were obtained from three independent experiments, the incubation with each test compound being performed in four different wells per experiment. The asterisk indicates significantly different from the solvent control value (100%; Dunnett's multiple comparison test, $p < 0.05$).

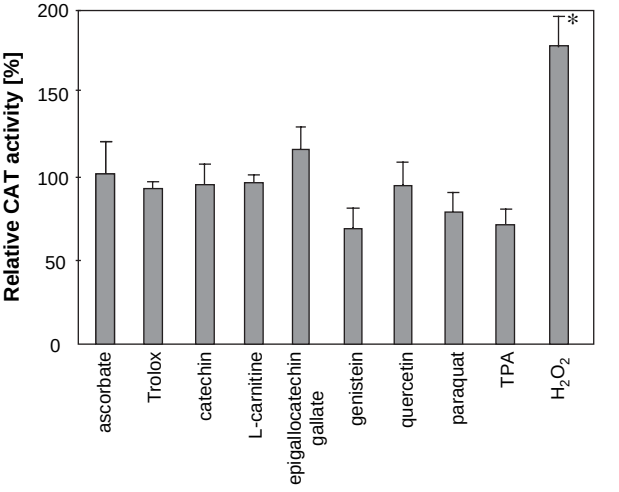

Figure 3. Effect of the test compounds on CAT promoter activity. The results are expressed as mean ± SD of the relative CAT promoter activities (i.e. the quotient of the CAT promoter activity in the presence of the test compound dissolved in DMSO and the CAT promoter activity in the presence of DMSO alone × 100). The concentration of each compound in the wells was: 500 μM AS, 10 μM TX, 50 μM CA, 1 mM LC, 50 μM EGCG, 5 μM GN, 10 μM QU, 100 μM PQ, 0.15 μM TPA and 50 μM HP. The data were obtained from three independent experiments, the incubation with each test compound being performed in four different wells per experiment. The asterisk indicates significantly different from the solvent control value (100%; Dunnett's multiple comparison test, $p < 0.05$).

promoter activity of the cells exposed to GN, QU and PQ being similar (i.e. 2.0-, 2.1- and 2.2-fold, respectively, when compared to DMSO-treated cells). None of the nine compounds tested was able to enhance the rat CAT promoter activity expressed in V79 cells (Figure 3). In order to make sure that the test system worked properly, V79 cells expressing the rat CAT promoter were exposed to 50 μM HP, which in the past has clearly been shown to enhance the expression of CAT in cultured cells [10]. As shown in Figure 3, exposure of V79 cells expressing the rat CAT promoter to a high HP concentration clearly enhanced the CAT promoter activity. Thus, the failure of AS, LC, CA, EGCG, GN, PQ, QU, TPA and TX to activate the CAT promoter is not due to an inherent limitation of the cell system used.

Discussion

The transfection of the human GPx1, the human SOD1 and the rat CAT gene promoter sequences into V79 cells was achieved by the calcium phosphate coprecipitation method. According to the literature [15] with this procedure a transfection efficiency of up to 50% can be reached, but in most studies [16] a 10–20% transfection efficiency has been reported. With the method described in the present study a very high transfection efficiency (60–80%) was achieved. When performing transient transfections the period of time in which the maximal expression of the transgene occurs is strongly limited due to the instability of the foreign DNA in the host cell [17,18]. Preliminary experiments had shown that with the method applied in this study the maximal expression of the luciferases occurred 24 h following transfection. Therefore, the test compounds were added immediately after the transfection had been accomplished and the relative GPx1, SOD1 and CAT activities were determined 24 h later, i.e. when the maximal transcription rate was expected.

When comparing the results of the reporter gene assay with data present in the scientific literature one should bear in mind that: (1) V79 cells are only incubated up to 24 h with the test compounds and some compounds may need longer incubation times to enhance the gene promoter activities; (2) the incubation of V79 cells with high concentrations of the test compounds can lead to overt cytotoxicity, so that these high concentrations cannot be tested; and (3) compounds, which are able to enhance the activity of antioxidative enzymes by mechanisms other than the increase in gene promoter activity (e. g. mRNA stabilization) may be overseen.

In the present study nine compounds were tested in V79 cells expressing the human GPx1, the human SOD1 and the rat CAT gene promoter sequences. For six of them (CA, EGCG, GN, PQ, QU and TPA) evidence for the ability to modulate/induce GPx1,

SOD1 and/or CAT activities, either *in vitro* or *in vivo*, has been published (see below). AS, LC and TX are three antioxidants for which no or no clear-cut evidence regarding their ability to modulate/induce the three above-mentioned enzymes has been presented.

In the skin of mice fed CA for 2–4 weeks CAT and SOD activities were reduced, while GPx activity was enhanced [19]. Furthermore, CA prevented the reduction of CAT and SOD activities in the skin of mice fed CA for 2–4 weeks and exposed to UV-B radiation [19]. In rat brain astrocytes [20] treated with 0.1 μM CA for 2–7 days and in the rat pheochromocytoma cell line PC12 [21] treated with 10–100 μM CA for 2–7 days SOD mRNA levels were increased. In the present study it was shown that CA does not result in an enhanced CAT, GPx1 or SOD1 gene promoter activity. Hence, CA most probably regulates the activities of the three above-mentioned enzymes by stabilizing the corresponding mRNAs or the translated enzymes.

An EGCG-rich green tea extract upregulated CAT and SOD activities in fruit flies [22]. In contrast, CAT and SOD activities remained unchanged when the human oral squamous cell carcinoma cell lines OSC2 and OSC4 as well as normal primary human epidermal keratinocytes were treated with 50 μM EGCG for 30 min [23]. In a recent study by Lee and Lee [24] it was shown that neuronal cell injury induced by advanced glycation endproducts is accompanied by a decrease in CAT and SOD activities and a concomitant increase in GPx activity and that EGCG counteracts these effects. In the present study EGCG did not lead to enhanced CAT, GPx1 and/or SOD1 gene promoter activities in V79 cells. Taken together, these results suggest that EGCG only modulates antioxidative enzyme activities when cells are 'stressed'. However, further experiments are needed to corroborate this assumption.

GN (100 μM) resulted in an increased GPx1 activity in the prostatic carcinoma cell lines LNCaP and PC-3 as well as in the colon adenocarcinoma cell line Caco-2 without affecting CAT and SOD activities [12,25]. However, as mentioned before, 50 and 100 μM GN enhanced CAT and SOD activities in the murine macrophage cell line RAW 264.7 [13]. In the present study it could be shown that 20 μM GN leads to an increase in the GPx1 and SOD1 gene promoter activities without affecting the CAT gene promoter. In this case one should bear in mind that the high GN concentrations used in the three above-mentioned studies (50–100 μM) were extremely cytotoxic to V79 cells and could therefore not be tested in the reporter gene assay.

Yoo et al. [26] reported that the herbicide PQ is capable of activating the SOD1 gene promoter in HepG2 cells. Furthermore, Röhrdanz et al. [27] demonstrated that the mRNA levels for CAT and SOD1 were enhanced in primary rat hepatocytes exposed for 20 h to 0.1–1 mM PQ. An increase in CAT and SOD activities accompanied by a decrease in GPx activity was observed in human gingival fibroblasts (Gin-1 cells) incubated with 0.25–2 mM PQ [28]. In the present study 100 μM PQ enhanced SOD1 as well as GPx1 gene promoter activities, whereas it did not affect the CAT gene promoter activity. The discrepancies with the cited studies remain at present unexplained. However, as in the case of GN it should be noted that higher concentrations of PQ could not be used, since they were cytotoxic to V79 cells.

On the one hand gavage administration of the natural flavonoid QU to rats for 2 weeks resulted in a decrease of CAT and GPx activities in erythrocytes [29]. On the other hand Kahraman and Inal [30] showed that QU prevented the decrease in CAT, GPx and SOD activities in erythrocytes and skin of rats irradiated with ultraviolet A light. The results from *in vitro* studies regarding the capacity of QU to induce antioxidative enzymes are contradictory. In the rat hepatoma cell line H4IIE 5–100 μM QU reduced GPx and SOD1 mRNA levels [31]. In the human hepatoma cell line HepG2 0.1–1 μM QU led to an increase in GPx mRNA levels and to a decrease in SOD1 mRNA levels, while 100 μM QU enhanced CAT as well as SOD1 mRNA levels [32]. In the present study the incubation of V79 cells with 10 μM QU resulted in a clear increase in GPx and SOD1 reporter gene activities. Treatment of the cells with higher QU concentrations (up to 100 μM) could not be tested because of the strong cytotoxicity of QU.

The tumour promoter TPA has been shown to enhance the SOD1 gene promoter activity in the human cervix carcinoma cell line HeLa [14] and in COS-7 cells [10]. In accordance with these results, TPA increased the SOD1 gene promoter activity, but this increase did not reach statistical significance. In the case of AS, LC and TX no experimental evidence that these compounds might induce CAT, GPx1 and/or SOD1 enzyme activities does in fact exist. Accordingly, in the present study none of these compounds resulted in an increase in CAT, GPx1 and/or SOD1 gene promoter activities.

Toyokuni et al. [10] had previously shown that HP in a concentration range of 1–100 μM enhanced the rat CAT promoter activity. Since none of the nine above-mentioned compounds led to such an effect, 50 μM HP was added to V79 cells expressing the rat CAT promoter to test whether the assay principally works. As in the case of COS-7 cells [10], HP led to an increase in CAT promoter activity in V79 cells, thus demonstrating that the reporter gene construct used in this study principally does respond to CAT inducers such as HP.

In conclusion, the data presented show that a reporter gene assay to prove the ability of natural

compounds to modulate the gene promoter activities of antioxidative enzymes in V79 cells has been established and that compounds such as GN, QU, PQ and HP can be used as positive controls in future screening programmes. The advantages of the reporter gene assay described in this report are that: (1) it is performed in a 96-well-plate format; hence, a high number of compounds and different concentrations of a single compound can be tested at the same time; (2) results are obtained within a very short period of time (3 days); and (3) animal testing is avoided. The reporter gene assay can also be run with other cell lines, whereby the transfection efficiency varies from cell line to cell line. In the case of Caco-2 and IEC-18 cells the transfection efficiency was 10 and $< 1\%$, respectively (A. Wiencierz, unpublished observations).

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