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Effect of Prooxidative Natural Products: Comparison of the OSI1 (YKL071w) Promoter Luciferase Construct from Yeast with an Nrf2/Keap Reporter System

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Abstract: The oxidative stress response (OSR) in yeast is under the control of oxidation-sensitive cysteines in the Yap1p transcription factor, and fusion of the Yap1p-dependent OS-induced promoter of the *YKL071w* gene (*OSI1*) to a luciferase coding sequence makes a sensitive reporter for OS induced by electrophiles. In mammalian cells, the OSR induced by electrophiles is coordinated in a mechanistically similar way via oxidation-sensitive cysteines in the kelch-like ECH-associated protein 1 (Keap1)– nuclear factor erythroid 2-related factor 2 / antioxidant response element (Nrf2/ARE) system. Many electrophilic oxidants have already been independently shown to trigger both the Yap1 and Keap1 systems. Here, we investigated the responses of Yap1 and Keap1 reporters to sulforaphane (SFN), allyl isothiocyanate (AITC), phenylethyl isothiocyanate (PEITC), previously known to stimulate Keap1–Nrf2/ARE but not known to activate Yap1, and as a positive control, allicin, previously reported to stimulate both Yap1 and Nrf2. We have compared the reciprocal responsiveness of the respective reporter systems and show that the yeast reporter system can have predictive value for electrophiles that stimulate the mammalian Keap1–Nrf2/ARE system.

Keywords: Yap1p; YKL071w; luciferase; allicin; phenylethyl isothiocyanate; allyl isothiocyanate; sulforaphane; monomethyl fumarate; diethyl fumarate; kavalactones; hepatocytes; electrophile; oxidative stress; antioxidant; phase II protective enzymes

1. Introduction

Cells defend themselves against oxidative stress by having oxidation-sensitive proteins, which, when triggered, lead to the expression of antioxidant genes. The oxidative stress response in yeast is coordinated by the oxidation-sensitive Yap1p transcription factor, which upon oxidation of critical cysteine thiols, accumulates in the nucleus and transcribes antioxidant phase II genes [1]. Electrophilic alkylating agents such as the phytochemical allicin or the xenobiotic *N*-ethylmaleimide oxidize C-term cysteines in Yap1p. In contrast, H_2O_2, working in conjunction with Gpx3p and Ybp1p, causes intramolecular folding by internal disulfide bridge formation between N- and C-term cysteines

in Yap1p. In both cases, the nuclear export protein binding site becomes masked, causing Yap1p to accumulate in the nucleus, thus enabling it to transcribe target genes [2,3]. In the course of our work with the sulfur-containing electrophile allicin (diallylthiosulfinate) that is produced as a defense substance by garlic (*Allium sativum*) upon tissue injury [4], we developed a reporter system for Yap1p activation by fusing the Yap1p-dependent promoter of the YKL071w open reading frame to a luciferase coding sequence [3]. A broad spectrum of oxidative and thiol-reactive substances induce the YKL071w gene, for example, allicin (27-fold) [5], the mycotoxin patulin (253-fold) [6], the phytochemical celastrol (31-fold) [7] and the pentose degradation product furfural (7-fold) [8]. We gave *YKL071w* the working name oxidative stress-induced1 (*OSI1*) [3].

Allicin has been shown to activate the Keap1–Nrf2/ARE system that, in mammalian cells, perceives electrophilic oxidants, and coordinates the OSR [9–11]. Keap1 (Kelch-like ECH-associated protein) in its reduced state has free cysteine thiols and associates with the transcription factor Nrf2, targeting it for ubiquitination and degradation in the cytosol. However, when the cysteine thiols in Keap1 are alkylated by electrophiles, Keap1 dissociates from Nrf2, which is thus stabilized and transported to the nucleus, where it associates with the co-transcription factor Maf and facilitates transcription of antioxidant phase II genes that have an antioxidant response element (ARE) in their promoter [12]. Reporter systems based on the Keap1–Nrf2/ARE response have been used to detect potentially useful electrophilic or pro-electrophilic drugs that activate phase II protective enzymes, and thus work physiologically as antioxidants [13]. For example, using ARE reporter systems, certain cancer chemotherapeutic agents were demonstrated to activate Nrf2 [14], and in the context of the role of oxidative stress in neurodegenerative disorders, various botanicals were shown to exert a protective effect via activation of ARE-regulated genes in astrocytes [15]. Another example is the LuSens assay system employing a human keratinocyte cell line transfected with an ARE::luciferase construct, which was developed to test for undesirable skin-sensitizing substances in response to EU legislation banning animal testing for cosmetic products [16,17]. In assays using the Keap1–Nrf2/ARE system, the phytochemical sulforaphane is usually employed as a positive control because it alkylates Keap1 efficiently and results in a large ARE-dependent response. Sulforaphane, alone or in combination with other substances, has been used to combat oxidative stress and reduce inflammation [18,19].

Because KEAP1 and Yap1 are both activated by the same chemical mechanisms, logically, if an electrophilic oxidant reacts with the sensitive cysteines in Yap1, it will most likely react chemically with the sensitive cysteines in Keap1, because in both systems these reactive cysteines have evolved to detect oxidative stress and coordinate the oxidative stress responses in their respective hosts. Indeed, very many such substances have already been independently shown to trigger both the Keap1 and Yap1 systems (Table 1).

Table 1. A selection of substances shown to activate both the Keap1–Nrf2/ARE and Yap1 oxidative stress response systems in mammals and in yeast, respectively.

Substance	Activation of Keap1–Nrf2/ARE	Activation of Yap1
Acrolein	Tirumalai et al. (2002) [20]	Ouyang et al. (2011) [21]
Cadmium	Chen and Shaikh (2009) [22]	Wemmie et al. (1994) [23]
Celastrol	Trott et al. (2008) [7]	Trott et al. (2008) [7]
Diamide	Piccirillo et al. (2009) [24]	Delauney et al. (2000) [25]
epigallocatechin gallate	Kanlaya et al. (2016) [26]	Maeta et al. (2007) [27]
hydrogen peroxide	Covas et al. (2013) [28]	Delauney et al. (2000) [25]
5-hydroxymethylfurfural	Ya et al. (2017) [29]	Kim and Hahn (2013) [30]
Methylglyoxal	Nishimoto et al. (2017) [31]	Maeta et al. (2004) [32]
Resveratrol	Ungvari et al. (2010) [33]	Escote et al. (2012) [34]

In the work reported here, we test the sensitivity of the OSI1::luciferase reporter in yeast and the Keap1–Nrf2/ARE reporter in murine hepatocytes, in direct comparison, for four test substances. Allicin is known to activate both Yap1 and Keap1–Nrf2/ARE, but conversely, phenylethyl isothiocyanate

(PEITC), allyl isothiocyanate (AITC), sulforaphane (SFN) are known to activate Keap1–Nrf2/ARE but it was unknown whether they activated Yap1. A congruence for response by the two reporter systems was shown in all cases. Furthermore, we tested the OSI1::luciferase reporter with monomethyl fumarate (MMF) and dimethyl fumarate (DMF), non-isothiocyanates but α-β-unsaturated electrophilic oxidants known to activate Keap1–Nrf2/ARE and 3 kavalactones (methysticin, yangonin, kavain) and a diterpene lactone (andrographolide), weak antioxidants which activate Keap1–Nrf2/ARE by a non-oxidative mechanism. We discuss the advantages offered by the yeast-based method compared to the animal cell method for large scale screening procedures.

2. Materials and Methods

2.1. Test Substances

Allicin was synthesized according to our published protocol [35] and used as an aqueous solution. PEITC was purchased from Alpha Aesar Ltd. (Karlsruhe, Germany), AITC, and SFN from Sigma-Aldrich (Darmstadt, Germany). All these substances are water-soluble at the concentrations used and were used as aqueous solutions in the OSI1 tests. However, because it is sometimes necessary to dissolve sparingly soluble substances in DMSO, tests with the ARE reporter system were carried out with solutions of the test substances in 0.03% DMSO. No effect of DMSO on cell viability or induction of luminescence was observed up to 0.1%.

2.2. Hepatocyte Cell Culture

Primary murine hepatocytes were isolated, as described previously [36]. The cells were cultured in DMEM High Glucose medium (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Madison, WI, USA) and 1% penicillin/streptomycin under normoxia at 37 °C. The cells were transfected with the SIN-lenti-ARE reporter gene plasmid that allows the measurement of Nrf2 activity by bioluminescence detection.

For CellTiter-Blue® (Promega, Walldorf, Germany), as well as ARE reporter gene assays, 3×10^4 cells/well were seeded in a 96-well microtiter plate. Cells were treated with either an aqueous solution of allicin, or PEITC, AITC, and SFN, in up to 0.1% DMSO for 6 h. The DMSO control corresponds to the highest concentration of DMSO used in the treatment.

The protocol was approved by the regional authorities of North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz LANUV North Rhine-Westfaphalia, Germany, ID: 8.87-51.05.20.10.063).

2.3. Cell Titer Blue® (CTB®) Assay

The CTB® assay (Promega) was conducted as recommended by the manufacturer without any changes to the protocol.

2.4. ARE Reporter Gene Assay

The SIN-lenti-ARE reporter gene construct [37] was based on the addgene plasmid #24308, in which the luciferase gene is under the control of an upstream minimal promoter driven by multiple TCF (T-cell factor) response elements. The 7 × TCF sequences of the 7TFP plasmid (a kind gift from Roel Nusse, Department of Developmental Biology, Howard Hughes Medical Institute, Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA, USA [38]) were removed by enzymatic restriction using PstI and NheI and replaced with the antioxidant response element (ARE) consensus sequence of the NADPH:quinone oxidoreductase 1 promoter (ggtaccagtctagagtcacagtgacttggcaaaatcgctagc) [19,39]. Test restriction, gel electrophoresis, and DNA sequencing verified the resulting plasmid DNA. The production of lentiviral particles was conducted as described before [40]. Briefly, HEK293 cells were cotransfected with a second-generation lentiviral plasmid system containing SIN-lenti-ARE as transfer vector, psPAX2 as a packaging vector,

and pMD2.G as the vector encoding for the envelope protein. Supernatants containing lentiviral particles were used for gene delivery.

Primary murine hepatocytes were transfected with the SIN-lenti-ARE reporter gene construct. Cells were lysed in passive lysis buffer (PLB) as recommended by the manufacturer (Promega). For the luciferase assay, 50 µL of the lysate was transferred to a white 96-well plate. Luciferase assay reagent I (LAR I) was injected automatically by the luminometer (Glomax 96, Promega). Sulforaphane (SFN: 7.5 µM) treatment was included as a positive control for Nrf2 activation. Measurement was conducted with default setting parameters (reagent volume 50 µL, delay 0.4 ms, integration time 10 s). Because sparingly soluble test substances often need to be dissolved in aqueous DMSO and then added to the test mix, a control with the highest DMSO end concentration but without test substance was performed.

2.5. Yeast Cell Cultivation

The BY4742 wildtype strain (Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) was used. Yeast cells were cultivated on CSM-medium (7 g L^{-1} yeast nitrogen base containing $(NH_4)_2SO_4$ without amino acids (ForMedium Ltd., Norwich, UK); 40 g L^{-1} glucose (Carl Roth, Karlsruhe, Germany); 0.8 g L^{-1} CSM complete dropout (ForMedium Ltd., Norwich, UK) at 28 °C and liquid culture was shaken at 220 rpm.

2.6. OSI1-Promoter::Luciferase Reporter Construct

Details of the cloning procedure have been published elsewhere [3]. The finished construct was amplified in *E.coli* DH5α and verified by colony PCR, restriction digestion, and sequencing the plasmid. It was transformed into BY4742 yeast cells by electroporation and plated out on uracil-lacking CSM medium.

Luciferin (Diagonal, Münster, Germany) was dissolved in 0.1 M sodium citrate, pH = 5 (Carl Roth, Karlsruhe, Germany) to a final concentration of 1 mM luciferin. Then, 100 µL of an overnight-culture in CSM-medium was adjusted to an OD_{600} = 0.5 and was treated with 25 µL of 5 × concentrated aqueous solution of test substance and 25 µL of luciferin-solution in black-sided, transparent-flat-bottomed 96-well microtiter plates. Luminescence was measured in a Tristar2S plate reader (Berthold, Bad Wildungen, Germany).

2.7. Statistics

Error bars show standard deviation. A normal distribution was assumed and a one-way ANOVA was performed with Dunett's multiple comparisons test for significance (** $p < 0.01$).

3. Results

Comparison of the ARE Reporter System in Murine Hepatocytes and OSI1 in BY4742 Yeast Cells

The four test substances, allicin, phenylethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC) and sulforaphane (SFN), were able to induce the luciferase reporter in both systems at low µM concentrations (Figure 1). In the ARE assay, which was measured after 6 h of co-cultivation, the concentration-dependent increase of the luminescence signal compared to non-treated controls ranged from ~2-fold (allicin) to ~4-fold (PEITC, AITC, SFN). Allicin showed significant ARE activation at 10 µM, PEITC at 2.5–5 µM, AITC at 5–10 µM, and SFN at 2.5–7.5 µM, respectively (Figure 1A,C,E,G). In comparison, after 1 h of co-cultivation with the four test substances, the OSI1 reporter in yeast cells showed a 15-fold (sulforaphane) to >50-fold induction (allicin) (Figure 1 B,D,F,H). The ARE reporter was more sensitive to sulforaphane (2.5 vs. 10 µM; Figure 1G,H) but the OSI1 reporter system was more sensitive to allicin (5 vs. 10 µM; Figure 1A,B) and PEITC (0.5 vs. 2.5 µM; Figure 1C,D).
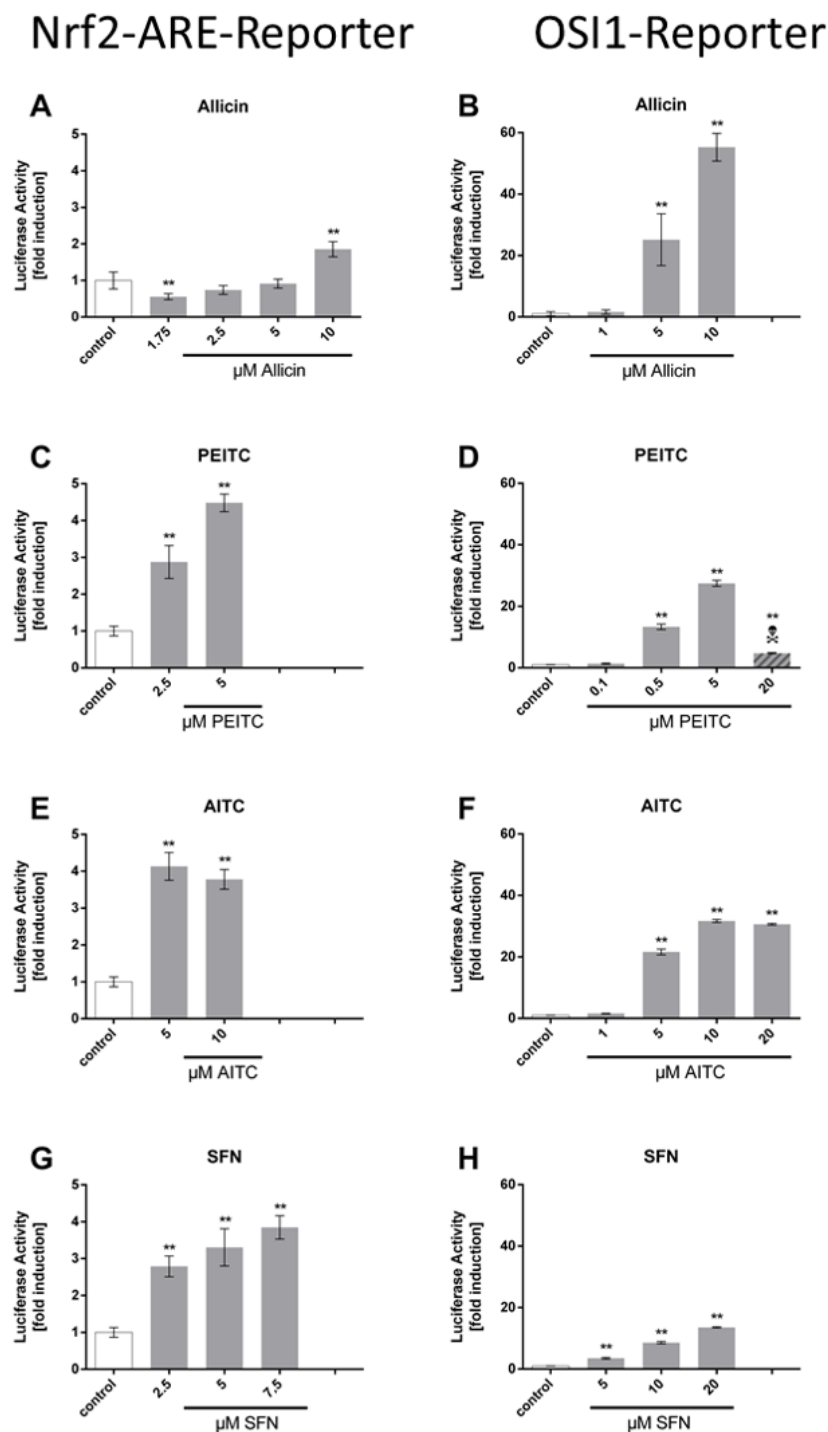
Figure 1. Responses of the antioxidant response element (ARE) and the OS-induced promoter of the YKL071w gene (OSI1) reporters to allicin, phenylethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC), sulforaphane (SFN). (**A,C,E,G**) show the ARE assay with murine hepatocytes transfected with the SIN-lenti-ARE gene construct as reporter. Measurements were taken 6 h after treatment (*n* = 8). (**B,D,F,H**) show the OSI1 assay with yeast cells transformed with the OSI1 promoter–luciferase gene construct as reporter (*n* = 3). Measurements are shown after 1 h of co-cultivation with the respective test substance. Attention is drawn to the different *y*-axis scales of the ARE and OSI1 tests. Error bars show standard deviation. A normal distribution was assumed, and a one-way ANOVA was performed with Dunett's multiple comparisons test for significance (** $p < 0.01$). RLU is the abbreviation for Relative Luminescence Units.

Routinely, in the ARE assay with murine hepatocytes, an "end point" measurement is made, in this case, after 6 h of co-cultivation of the cells with the test substance. However, for the OSI1 reporter, no pre-cultivation of the yeast cells with the test substance is necessary, as the measurements in the 96-well plates are dynamic from time zero and show the increase in luminescence over time (Figure 2), i.e., a kinetic is established rather than an end-point measurement. Thus, it can be seen that within the first 30 min of exposure, all the test substances activated the OSI1 reporter in the low μM range. The OSI1 reporter was particularly responsive to PEITC (0.5 μM) and allicin (1 μM), while AITC and SFN showed clear responses in the 5–10 μM range (Figure 2A–D). The data from the measurements at the 1 h time point were incorporated into Figure 1B,D,F,G to facilitate a more direct comparison with the ARE reporter system.
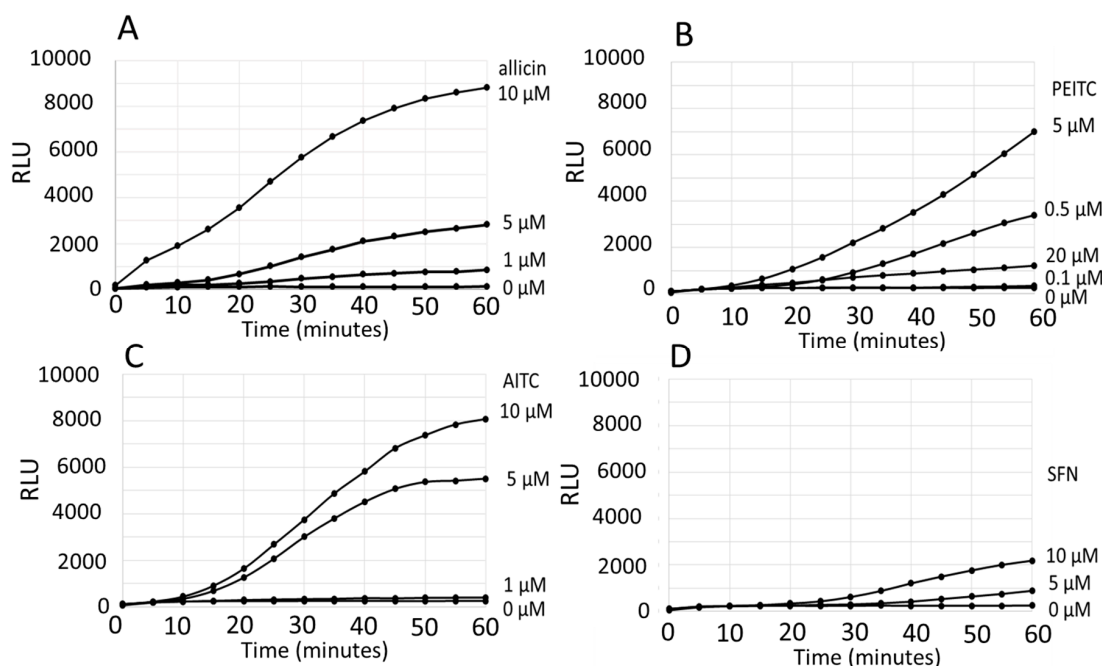
Figure 2. Dynamic response of yeast cells containing the *OSI1*::luciferase reporter to (**A**) allicin, (**B**) phenylethyl isothiocyanate (PEITC), (**C**) allyl isothiocyanate (AITC), (**D**) sulforaphane (SFN). Dose-dependency can be seen, and higher concentrations, which can already potentially damage the cells, show a lower luminescence signal, as can be seen in the example of 20 μM PEITC (**B**). RLU is the abbreviation for Relative Luminescence Units.

With the exception of the thiosulfinate allicin, all of the substances tested in the previous section were isothiocyanates. We, therefore, screened the non-isothiocyanate α-β-unsaturated electrophilic substances monomethyl and dimethyl fumarate for activity with the *OSI1*::luciferase reporter. The response was measured over 6 h, and the dose-dependent kinetics of the *OSI1*::luciferase response is clearly visible (Figure 3). Interestingly, the OSI1 test system responded more rapidly to allicin than to mono- and dimethyl fumarate, and this emphasizes the advantage of the *OSI1*::luciferase reporter giving a kinetic response, compared to the ARE reporter which only gives a single time point reading.

Kavalactones are weak antioxidants present in the rhizome and roots of the kava plant (*Piper methysticum* G. Forst), which were demonstrated to activate Keap1–Nrf2/ARE by a non-oxidative route [41]. Thus, we would not expect them to stimulate the oxidation-sensitive trigger-cysteines of Yap1p and activate the OSI1::luciferase reporter. This was indeed the case and analytically pure samples of methysticin, yangonin, kavain and the diterpene lactone andrographolide [42] did not elicit luminescence up to a concentration of 400 μM (DMSO 5% (*v/v*); data not shown). This emphasizes that the Yap1p-dependent OSI1::luciferase reporter detects physiologically active electrophilic oxidants and

not substances that activate Keap1–Nrf2/ARE by a non-oxidative route, for example, by affecting the binding pocket of the Keap1–Nrf2 complex.
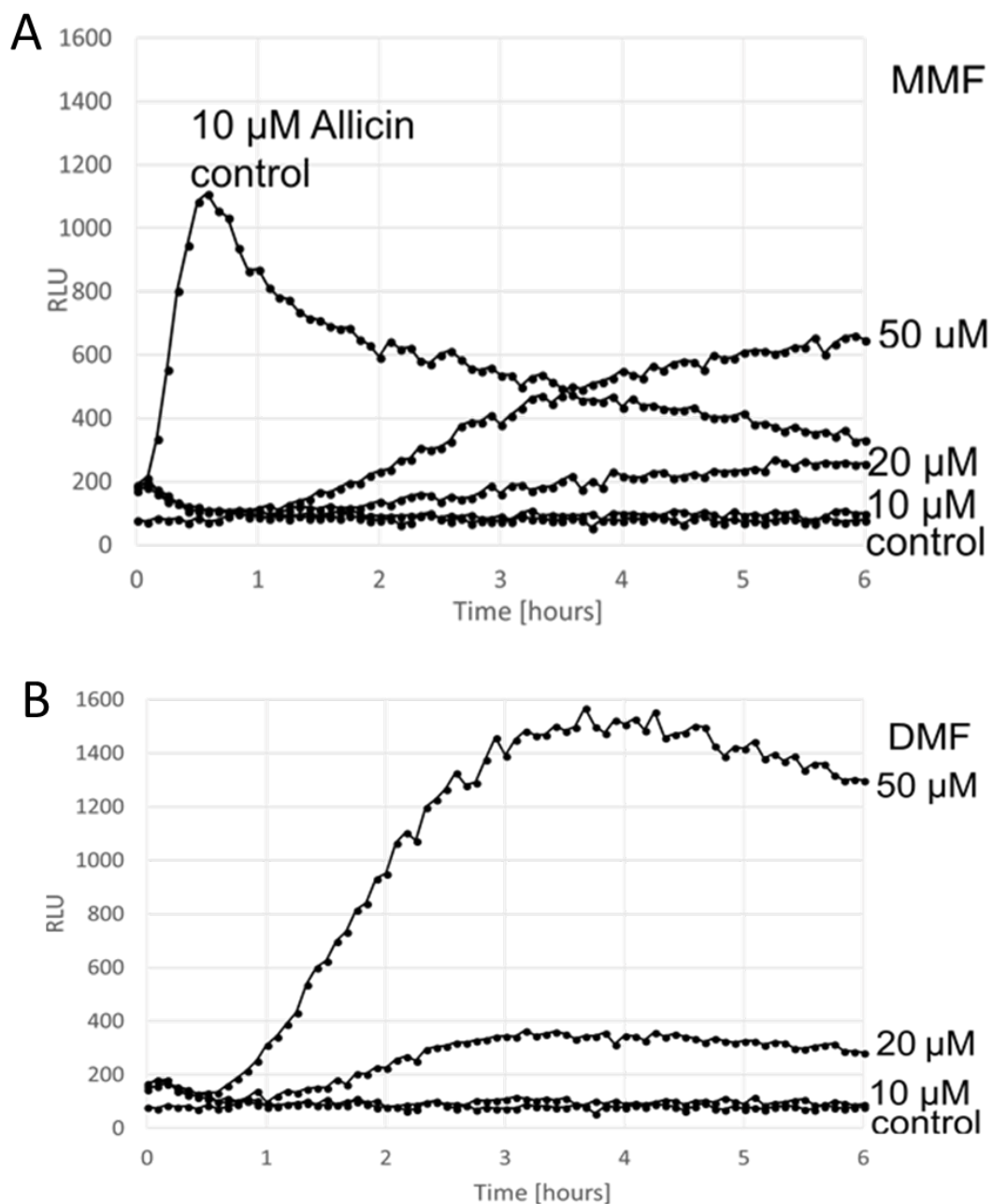

Figure 3. Dynamic response of yeast cells containing the OSI1::luciferase reporter to (**A**) monomethyl fumarate (MMF) (**B**) dimethyl fumarate (DMF). MMF and DMF were dissolved in DMSO. In the assay was 5% (*v/v*). *n* = 3. RLU is the abbreviation for Relative Luminescence Units.

4. Discussion

In this study, different substances were tested that are either known to be thiol-reactive (as is the case for thiosulfinates and isothiocyanates) or known to induce the Nrf2 system without oxidizing or modifying the relevant cysteine residues. An overview of the chemical structures is given in Figure 4.
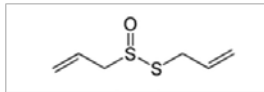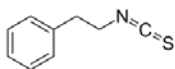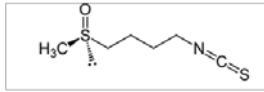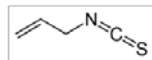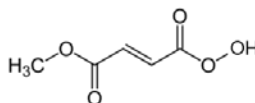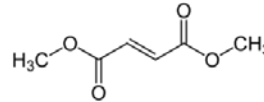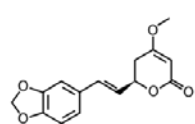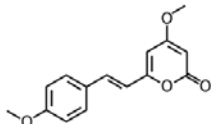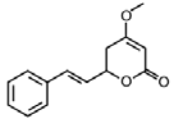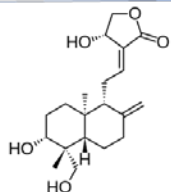
Figure 4. Overview of the chemical structure of the tested compounds. The compounds can be roughly divided into five chemical groups. Allicin belongs to the thiosulfinates, which are typical for representatives of the genus *Allium*. The isothiocyanates AITC, PEITC, and sulforaphane belong to the isothiocyanates and are mainly found in the Brassicaceae family. Methyl fumarates are esters of fumaric acid and are synthetic in nature. All these compounds have a thiol reactivity in common. The kavalactones (methysticin, yangonin, kavain) and the diterpene lactone andrographolide are derived from the plant *Piper methysticum* G. Forst and activate the Keap/Nrf2 system in a non-oxidative way.

Keap1–Nrf2/ARE-based assays represent an elegant and relatively easy-to-use approach to examine the oxidative potential of electrophilic substances on mammalian cells [16]. These assays are usually conducted with immortalized human or murine cell lines, thereby allowing high-throughput screening. However, these are cancer cell lines originating mostly from tumor biopsies. In the last few years, research has shown that, aside from its well-known antioxidant functions, Nrf2 has roles in the development and the progression of cancer. Furthermore, it has been shown that Nrf2 is also involved in many oxidative stress-independent intracellular processes, such as the cell cycle [40], metabolism [43,44], and differentiation [45]. Tumor cells have been shown to highjack the Nrf2/ARE system to adjust cellular processes to their specific requirements [46]. Moreover, many human solid cancers, as well as commonly used cell lines, show gain-of-function mutations of Nrf2 or loss-of-function mutations of Keap1 [47,48]. All these aspects make the use of cancer cell lines for Keap1–Nrf2/ARE-based assays at least questionable. For these reasons, we used freshly isolated primary cells in our study to better represent physiological conditions. In this case, the ARE-based assay requires the keeping and sacrifice of animals to obtain test cells, which are then transfected with ARE reporter construct before an assay. In the standard ARE protocol, from the onset of an assay with test substances, a six-hour incubation period is used and gives a point reading as output. Furthermore, the ARE procedure requires a prior assessment of the test substances for their effects on cell viability (e.g., CTB®) to decide on a suitable working concentration. In contrast, an overnight culture of yeast cells, already stably transformed with the OSI1 reporter construct, provides the starting inoculum for the log-phase cells used for the OSI1 reporter assay. The OSI1 reporter assay gives a continual output after the addition of test substances over any chosen concentration- and time-range, allowing for kinetic observations. Furthermore, the plate reader allows parallel measurements of luminescence and cell growth kinetics. Concentrations of test substance which reduce yeast cell growth show a reduced luminescence response compared to lower concentrations of the test substance. Thus, dose-dependency can be seen up to concentrations that begin to inhibit growth.

Furthermore, because cell culture itself is stressful, there is some induction of the ARE protection system leading to relatively high background luminescence in the controls. Thus, the OSI1-reporter has a further advantage in that it shows a several-fold higher signal-to-noise ratio in comparison to the ARE reporter and has a higher dynamic resolution.

In contrast to Keap1–Nrf2/ARE, the OSI1 reporter does not rely on slow translation events to accumulate the reporting transcription factor in the nucleus; therefore, its response is generally much faster, and the reaction to the stimulating substance can be followed easily in real-time. This enables the investigation of important kinetic parameters such as the uptake rate of the investigated substances or the bioavailability. The sensitivities of the ARE reporter and OSI1-reporter systems are similar for the tested substances and lie in the low μM range in both cases.

It should be emphasized, however, that the yeast system with the OSI1 reporter will mirror the Nrf2–Keap1/ARE system only with electrophilic oxidants that can attack trigger-cysteines in the respective sensor proteins. Substances that activate Nrf2 by non-oxidative mechanisms, such as the kavalactones and andrographolide, would not be expected to activate the OSI1 reporter, and indeed we show this to be so (Figure 3). Furthermore, because of differences in metabolism, yeast cells may, of course, be unable to modify some electrophilic compounds to convert them into active thiol oxidants as might happen in mammalian cells.

Nevertheless, the OSI1 reporter system offers a quick, sensitive, economical alternative to the ARE reporter system for detecting physiologically active electrophilic oxidants and has the added bonus that it does not require animal sacrifice. Indeed, a recent report also drew attention to similarities between the sensing of electrophiles in fungi via Yap1p activation and the Keap1–Nrf2/ARE in mammals [49].

An added advantage of working with yeast is that having identified an active substance, knock-out libraries of non-essential yeast genes and libraries maintaining knock-outs of essential genes as heterozygotes in diploid yeast lines are available for screens (www.euroscarf.de/index.php?name= News) to identify genes of interest related to the effects of the test substance [50].

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