The nuclear factor κB inhibitor (E)-2-fluoro-4′-methoxystilbene inhibits firefly luciferase

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Synopsis

Photinus pyralis (firefly) luciferase is widely used as a reporter system to monitor alterations in gene promoter and/or signalling pathway activities in vitro. The enzyme catalyses the formation of oxyluciferin from D-luciferin in an ATP-consuming reaction involving photon emission. The purpose of the present study was to characterize the luciferase-inhibiting potential of (E)-2-fluoro-4′-methoxystilbene, which is known as a potent inhibitor of the NF-κB (nuclear factor κB) signalling pathway that is used to modulate the NF-κB signalling pathway in vitro. Results show that (E)-2-fluoro-4′-methoxystilbene effectively inhibits firefly luciferase activity in cell lysates and living cells in a non-competitive manner with respect to the luciferase substrates D-luciferin and ATP. By contrast, the compound has no effect on Renilla and Gaussia luciferases. The mechanism of firefly luciferase inhibition by (E)-2-fluoro-4′-methoxystilbene, as well as its potency is comparable to its structure analogue resveratrol. The in vitro use of trans-stilbenes such as (E)-2-fluoro-4′-methoxystilbene or resveratrol compromises firefly luciferase reporter assays as well as ATP/luciferase-based cell viability assays.

Key words: firefly, luciferase inhibitor, nuclear factor κB, reporter gene assay, resveratrol


INTRODUCTION

Luciferase reporter systems are widely used reporter genes that allow for an easy luminescence detection of the activity of gene promoters and/or transcriptionally relevant signalling pathways in response to a variety of stimuli and modulators. Among the different luciferases known, Photinus pyralis (firefly) luciferase was the first to be cloned in 1985 [1] and is still most widely used. In a two-step reaction, the second of which is coupled to photon emission, firefly luciferase converts its substrate D-luciferin into oxyluciferin in an ATP- and oxygen-consuming reaction. For a detailed description of the chemistry of luciferase-catalysed reactions, see [2–5]. The luciferase reaction is also used in cytotoxicity/cell viability assays based on assessing cellular ATP levels. Often, coenzyme A is also present in firefly luciferase assay buffers as a light stabilizer due to its ability to perform thiolysis of dehydroluciferyl-AMP, a product of the luciferase reaction capable of inhibiting the enzymatic reaction; e.g. see [5] and references therein.

A number of chemical substances have been described which inhibit firefly luciferase activity by either competitive or non-competitive action. For example, different classes of firefly luciferase inhibitors and their mechanisms of action are discussed in a recent review by Leitao and Esteves da Silva [3], and additional firefly luciferase inhibitors were identified by Auld et al. [6]; as one might expect, various analogues of the substrate luciferin as well as structurally related benzothiazoles inhibit the reaction in a competitive manner [3,6,7]. ATP analogues are also capable of inhibiting firefly luciferase activity [6,8]. The luciferase-inhibitory potential of other classes of chemicals is less self-evident: among others, a number of alcohols, small alkanes, fatty acids, quinoline analogues, substituted benzylamides, different halogenated compounds used as general anaesthetics and certain ionic liquids also interfere with the activity of firefly luciferase [3,6,9,10], as well as the p53 inhibitor pifithrin-α [11] and the...
widely used antioxidant 3,5,4′-trihydroxy-trans-stilbene, better known as resveratrol [12]. Resveratrol is a potent non-competitive inhibitor of firefly luciferase with a reported $K_i$ value of $\sim 2 \mu M$ [12]. The authors of the latter paper stress that luciferase-based analyses of resveratrol-treated cells (e.g. resveratrol is often used for the assessment of antioxidative effects in cell culture) might be biased by the direct luciferase-inhibiting action of the compound. Of course, the same holds true for other luciferase inhibitors if used in cell culture for a purpose different from luciferase activity.

In the present study, we demonstrate that (E)-2-fluoro-4′-methoxystilbene {also known as NFκB-IA4 [NF-κB (nuclear factor κB) activation inhibitor 4]; Figure 1A}, a resveratrol analogue without antioxidative properties used as a specific inhibitor of NF-κB activation [13], inhibits firefly luciferase, but not other luciferases, in vitro with a potency comparable with resveratrol. In contrast with resveratrol, inhibition of firefly luciferase by NFκB-IA4 is sustained for >24 h in living cells, much longer than inhibition by resveratrol.

**MATERIALS AND METHODS**

**Chemicals and reagents**

NFκB-IA4 (catalogue no. 481412; Merck) and resveratrol (catalogue no. R5010; Sigma) were dissolved in DMSO in concentrations up to 20 mM and stored at $–20^\circ C$ for no longer than 4 weeks before use. Cell culture media, supplements and transfection reagents were purchased from Invitrogen. Chemicals for the preparation of luciferase assay buffers were purchased from PJK. D-Luciferin-ethylester was from Gentaur and firefly luciferase was from Roche; passive lysis buffer for luciferase assays...
was purchased from Promega. All other chemicals were purchased from Merck, if not otherwise indicated.

Cell culture and treatment
Mouse hepatoma cell lines Hepa1c1c7, 70.4 and 55.1c were grown in DMEM (Dulbecco’s modified Eagle’s medium)/F-12 medium supplemented with 10% fetal calf serum and antibiotics at 37°C and 5% CO2 in a humidified atmosphere. Cells were seeded at a concentration of 50000 cells/cm² 24 h prior to transfection and treated with the indicated concentrations of the compounds 24 h later. Concentration of the solvent DMSO was limited to 0.1% in all assays except for the in vivo monitoring of firefly luciferase activity, in which 1% DMSO was present.

70.4- and 55.1c-derived subclones stably transfected with the artificial β-catenin-driven firefly luciferase reporter plasmid STF (SuperTopflash) [14] and a plasmid-mediating resistance against G418 (pSV2neo; BD Biosciences) were routinely grown in medium additionally supplemented with 400 μg/ml G418 [15]. The selection antibiotic was removed from the cultures when plating them for experiments. Stably transfected cell lines are referred to as 70.4STF K15, 70.4STF K31 (both 70.4-derived), and 55.1cSTF K16, K60, K53 and K65 (55.1c-derived).

Cell viability assays
Cell viability/cytotoxicity of all compounds was analysed by the Neutral Red uptake and Alamar Blue assays using standard methodology. All concentrations used for cell treatment in the presented experiments did not cause any significant alterations in cell viability. Analyses with the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) based on the detection of cellular ATP levels by a firefly luciferase-dependent reaction (modified Ultra-Glo recombinant luciferase) were performed as recommended by the manufacturer.

Transfections
Cells were transfected with the STF reporter plasmid (see above), the CMV (cytomegalovirus) promoter-driven Renilla luciferase expression plasmid pRL-CMV (Promega), or an AP-1-responsive Gaussia luciferase expression vector [16] using LipofectamineTM 2000 according to the manufacturer’s instructions. Stably transfected cells derived from the 55.1c cell line were established as recently described [15].

Luciferase activity assays
Firefly luciferase activity was determined in a 96-well plate reader (Victor3V; PerkinElmer) as described previously [17] using a reaction buffer containing 20 mM tricine, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT (dithiothreitol), 270 μM co-enzyme A, 470 μM D-luciferin and 530 μM ATP at pH 7.8 [18]. Renilla luciferase reaction buffer contained 220 mM K3PO4, 1.1 M NaCl, 2.2 mM EDTA, 0.44 g/l BSA, 1.3 mM NaN3 and 1.43 μM coelenterazine at pH 5.0 [19]. The same buffer was used for measurement of Gaussia luciferase with the only modification that it contained 5.72 μM coelenterazine. Then 10 μl of cell lysate (firefly, Renilla; prepared in 1× passive lysis buffer) or cell culture medium supernatant (Gaussia) was mixed with 50 μl of the respective reaction buffer. Renilla buffer was added to the lysate/firefly reaction buffer mix after measurement of firefly luciferase activity. Luminescence was measured for a period of 10 s. For analysis of decay rates of the enzyme reaction, luminescence was continuously monitored for 9 min after addition of the reaction buffer. If luminescence counts are presented in the Figures instead of relative luciferase activity, counts/s are shown.

In the case of the addition of substances dissolved in DMSO to one of the luciferase assay buffers, DMSO concentration was limited to 5%. The firefly luciferase stock solution was prepared by dissolving 1 mg of the protein in 1 ml luciferase assay buffer, without ATP and D-luciferin, supplemented with 0.1% BSA.

Monitoring of luciferase in living cells was performed similar to [20] using a buffer containing 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 100 μM D-luciferin-ethylester and 1% DMSO. Cells were pre-incubated with 20 μM NFκB-BAI4 in culture medium for 30 min at 37°C and 5% CO2 followed by washing with PBS. Then, pre-warmed (37°C) assay buffer was added to the cells and luciferase signals were assessed after an additional 5 min of incubation in the plate reader or by the use of a CCD (charge-coupled device) camera (Raytest) using a time frame of 4 min (plate reader) or 8 min (CCD camera).

For the calculation of relative luciferase activity values, luminescence counts for each well were normalized to its corresponding cell vitality, as determined by the Alamar Blue assay, prior to cell lysis. Cell vitality-normalized luciferase activities are given relative to cell vitality-normalized values of untreated cells (percentage of control).

DNA isolation and real-time RT–PCR (reverse transcription–PCR)
Isolation of total RNA, RT by avian myeloblastosis reverse transcriptase (Promega), and real-time RT–PCR on a LightCycler instrument by the use of the FastStart DNA Master SYBR Green I kit (Roche) have been described recently [21]. Target gene expression was normalized to 18S rRNA expression according to [22]. PCR primers were as follows: Axin2_fwd, 5’-CGACGAATCGACGACATT-3’; Axin2_rev, 5’-TCCAGAC- TATGCCGCTTCC-3’; 18S rRNA_fwd, 5’-CGGCTACCACATCCAAAGAA-3’; 18S rRNA_rev, 5’-GCTGGAATTACGC- GCCT-3’.

RESULTS AND DISCUSSION

Inhibition of firefly but not Renilla or Gaussia luciferases by NFκB-BAI4
In a series of experiments aimed at analysing a possible interplay of β-catenin signalling and other cellular signalling pathways, the murine hepatoma cell lines Hepa1c1c7, 70.4 and 55.1c were transiently transfected with the β-catenin-driven firefly luciferase
reporter STF and treated with 20 μM NFxBAI4 for 24 h. As shown in Figure 1B, NFxBAI4 treatment caused an unexpected strong decrease of luminescence in all three cell systems. A similar inhibitory effect of NFxBAI4 was observed when a number of stably STF-transfected cell clones derived from the 70.4 or 55.1c cell lines were treated with the compound (Figure 3B). This was, however, not accompanied by a decrease in mRNA levels of the known β-catenin target genes Axin2 and Gpr49 (G-protein-coupled receptor 49) [15], thus casting doubt on a true inhibition of the pathway (Figure 1C), especially as a considerable concomitant reduction of target gene expression and reporter activity can be achieved by transfection of siRNA (small interfering RNA) directed against β-catenin mRNA (results not shown). One possible explanation for this discrepancy of β-catenin-dependent firefly luciferase reporter and target mRNA data was that NFxBAI4 causes an inhibition of the firefly luciferase reaction. To test this hypothesis, 20 μM NFxBAI4 was added directly into lysates of untreated Hepa1c1c7, 70.4 and 55.1c cells which had been transiently transfected with expression vectors for the firefly, Renilla or Gaussia luciferase 24 h before. NFxBAI4 strongly inhibited firefly luciferase signals in all cell lines, whereas the other luciferases, i.e. Gaussia and Renilla luciferase, were not affected (Figure 1D), demonstrating the specificity of NFxBAI4 for firefly luciferase. Pure commercially available firefly luciferase was also inhibited by NFxBAI4, demonstrating that the observed effects are not due to other components present in cell lysates (Figure 1E). Inhibition of firefly luciferase activity was further monitored in living luciferase-expressing cells incubated with a buffer containing the cell-permeable D-luciferin derivative D-luciferin-ethylester [20] following exposure of the cells to NFxBAI4 (Figures 1F and 1G), demonstrating the specificity of NFxBAI4 for firefly luciferase.

Using a commercially available cell viability assay based on the detection of cellular ATP levels by a modified firefly luciferase (Ultra-Glo recombinant luciferase, derived from the firefly Photinus pennsylvanicus; Promega), the inhibition of luciferase activity by NFxBAI4 was also detectable (Figure 1H). However, much higher concentrations of NFxBAI4 were needed for the inhibition of Ultra-Glo luciferase (IC₅₀≈200 μM), as compared with conventional firefly luciferase.

Comparison with the structurally related firefly luciferase inhibitor resveratrol

It has been reported that resveratrol, structurally related to NFxBAI4, inhibits firefly luciferase [6,12]. We thus compared the ability of both compounds to inhibit firefly luciferase activity derived from lysates of untreated firefly luciferase-expressing cells (Figure 2, and Supplementary Figure S1 at http://www.bioscirep.org/bsr/032/bsr0320531add.htm). The inhibitory potency of both substances was very similar in all four cell lines analysed, with IC₅₀ values of ~1 μM (Table 1). Almost identical concentration-dependent inhibition (IC₅₀≈1 μM) of firefly luciferase by NFxBAI4 was detected when a reaction buffer without coenzyme A was used (results not shown; for comparison, see also results in Figure 3E). Values obtained with resveratrol were comparable with a previously reported IC₅₀ value of ~2 μM [12].

We further compared the luciferase-inhibiting ability of NFxBAI4 and resveratrol in cells after different periods of incubation with 20 μM of the respective substance. NFxBAI4 strongly inhibited firefly luciferase after 1 h of incubation and the effect was still pronounced after 24 h (Figures 3A and 3B). Following 48 h of incubation with NFxBAI4, the inhibitory effect was still visible in 70.4-derived, but not in 55.1c-derived, cell lines (Figure 3C). By contrast, inhibition of firefly luciferase activity by resveratrol was weaker after 1 h and disappeared in all cell lines already at 24 h after addition of the compound to the cells (see Supplementary Figure S2 at http://www.bioscirep.org/bsr/032/bsr0320531add.htm). A possible explanation for this dissimilar behaviour of the two substances which share an equal potency of firefly luciferase inhibition directly after addition.

Table 1 IC₅₀ values for firefly luciferase inhibition by NFxBAI4 and resveratrol

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (μM)</th>
<th>NFxBAI4</th>
<th>Resveratrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>70.4STF K15</td>
<td>0.85</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>70.4STF K31</td>
<td>0.88</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>55.1cSTF K53</td>
<td>0.95</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>55.1cSTF K65</td>
<td>1.42</td>
<td>1.30</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2 Dose-dependency of firefly luciferase inhibition by NFxBAI4 and resveratrol

Dose-effect curves of luciferase inhibition by NFxBAI4 (A) or resveratrol (B) are shown in lysates from untreated 70.4STF K15 and 55.1cSTF K65 cells with stable expression of firefly luciferase. Then 20 μM NFxBAI4 was added to lysates of untreated cells 5 min prior to measurement. Means ± S.D. (n = 4) are given. Inhibition of firefly luciferase activity in lysates derived from other cell lines is depicted in Supplementary Figure S1 at http://www.bioscirep.org/bsr/032/bsr0320531add.htm.
Luciferase activity was assessed in lysates from various cell lines, derived from 70.4 or 55.1c mouse hepatoma cells, with stable expression of firefly luciferase. Then 20 μM NFxBAI4 was added to cell cultures and incubated for 1 h (A), 24 h (B) or 48 h (C) prior to lysis and measurement. Luciferase signals were normalized to cell vitality, as determined by the Alamar Blue assay. Means ± S.D. (n = 3 and 4) are given; *p < 0.05. For comparison see data for firefly luciferase inhibition by resveratrol in Supplementary Figure S2 at http://www.bioscirep.org/bsr/032/bsr0320531add.htm. (D) Dose-response analysis of firefly luciferase activity for the substrates ATP and D-luciferin, similar to the analyses performed with resveratrol [12]. A mixed hyperbolic equation was fitted to the data (Figure 3D). The results appear almost identical with those reported for resveratrol [12], demonstrating that firefly luciferase is inhibited by NFxBAI4 in a non-competitive manner, comparable with resveratrol (Figure 3D).

We further analysed whether NFxBAI4 would affect the decay of the luminescence signal. For this purpose, lysates were treated with 1 μM NFxBAI4 (equivalent to the IC50 of the compound). Analyses were performed in the absence of coenzyme A. As expected, an overall decreased intensity of the luminescence signal was observed when the inhibitor was present (Figure 3E). After a rapid initial decay of the signal during the first 2 min, signal intensity declined more slowly during the rest of the observation period when the untreated lysate was used, whereas the decrease appeared to be somewhat less pronounced in the presence of NFxBAI4 (Figure 3E).

**Improvement of a dual luciferase assay buffer by NFxBAI4**

An inhibition of firefly luciferase might be desired under certain conditions: for example, dual luciferase assays normally consist of an analysis of firefly luciferase activity followed by the assessment of Renilla luciferase activity in the same cell lysate. This implies that the activity of firefly luciferase is effectively quenched before measuring luminescence produced by the Renilla enzyme, an issue that is solved by a change of buffer composition and pH in the reaction tube. Using our buffers, residual activity of firefly luciferase is ~0.02% of the initial activity, as determined by measuring lysates containing only firefly but not Renilla luciferase before and after addition of the Renilla buffer (Figure 4A). The addition of 20 μM NFxBAI4 to the Renilla buffer further improved its ability to quench the firefly signal (Figure 4A), but did not influence Renilla luciferase activity, as determined by the addition of 20 μM NFxBAI4 to the Renilla luciferase reaction buffer in a classic dual luciferase assay with transiently transfected Hepa1c1c7 cells (Figure 4B). Thus, in principle, dual luciferase systems might be improved by addition of a firefly luciferase inhibitor to the Renilla luciferase reaction buffer.

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Conclusions

The potential of NFκB to inhibit firefly luciferase is important to know when conducting luciferase reporter analyses in lysates from cells treated with the compound, since these analyses might be compromised by the direct action of NFκB on the luciferase enzyme. The same applies to cell vitality assays that make use of firefly luciferase to determine the levels of cellular ATP as an indicator of metabolically active intact cells. In accordance with previous work [6, 12], we found that the structurally related molecule resveratrol also inhibits firefly luciferase, in a manner comparable with NFκB. Moreover, the glutamate receptor antagonist SIB-1893 [26], a trans-stilbene like NFκB and resveratrol, has been shown to inhibit firefly luciferase with a somewhat lower efficacy than NFκB [6] (please note that the compound is erroneously depicted as a cis-stilbene in the latter paper). Thus, it seems likely that trans-stilbenes constitute a structural class of firefly luciferase-inhibiting molecules which, however, has not been clearly identified as such in previous studies.

The problems caused by conducting luciferase assays in the presence of NFκB can be circumvented by using different luciferases (e.g. Gaussia luciferase). Another alternative might be the modified Ultra-Glo luciferase, which is present in Promega’s cell viability assay kit. The latter enzyme was inhibited by NFκB in our analyses, but only at very high concentrations of the compound which exceed the concentrations routinely used in cell culture. Although a direct quantitative comparison with the degree of inhibition obtained with the wild-type is impeded by the different reaction conditions of the assays, it is highly plausible that the modified enzyme is more resistant against inhibition by NFκB, as it is generally more resistant with respect to several other luciferase inhibitors [27]. This idea is supported by data presented on the Promega Corporation web page demonstrating that Ultra-Glo luciferase is more resistant against inhibition by the NFκB trans-analogue resveratrol (http://www.promega.com/de-de/resources/scientific_posters/compound-interference-of-celltiterglo-vs-pe-atplite-1step-poster/).

REFERENCES

NFκBAI4 inhibits firefly luciferase


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SUPPLEMENTARY ONLINE DATA

The nuclear factor κB inhibitor (E)-2-fluoro-4′-methoxystilbene inhibits firefly luciferase

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Figure S1 Inhibition of firefly luciferase by NFκBIA4 (A) or resveratrol (B) in additional cell lines, for the determination of IC₅₀ values shown in Table 1 in the main paper
For details please refer to the legend to Figure 2 in the main paper.

Figure S2 Inhibition of firefly luciferase by 20 μM resveratrol in stably transfected cell lines following incubation for 1 h (A) or 24 h (B), for comparison with Figures 3(A)–3(C) in the main paper
The effects of resveratrol, as compared with NFκBIA4, are less pronounced after 1 h and disappear after 24 h of incubation. Means ± S.D. (n = 3) are given; *P < 0.05.

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