Firefly luciferase inhibition: a widely neglected problem

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Numerous research groups are engaged in the analysis of the interference of plant constituents with the growth and survival of cancer cells. Among others, the polyphenolic trans-stilbene resveratrol and various flavonoids are extensively studied in this context. These compounds are generally thought to act in a cytoprotective, anti-tumorigenic manner, probably via their antioxidant and anti-inflammatory properties or by their ability to enhance the cytotoxicity of anticancer drugs. The underlying molecular details are addressed by an increasing number of publications, often by tracing back alterations in biological endpoints, e.g., apoptosis, to a substance-dependent disturbance of distinct cellular signaling pathways and transcription factors. For a recent review of resveratrol effects on the activity of various transcription factors, see Whitlock and Baek (2012). Luciferase-based reporter assays are among the most frequently used experimental tools to analyze the activity of downstream transcription factors of interest.

Unfortunately, an important confounder of luciferase reporter assays is still neglected by most researchers in the field. As many other enzymes, *Photinus pyralis*-derived firefly luciferase is subject to inhibition by chemicals acting as competitive or non-competitive inhibitors of the protein's catalytic activity, as reviewed by Auld et al. (2008) and by Leitao and Esteves da Silva (2010). In a routine assay setup, direct inhibition of the reporter enzyme by a substance present in the cell lysate cannot be easily distinguished from an inhibition of reporter gene transcription, if proper controls are missing. This is of pivotal importance for the research related to nutritional effects on the

growth and survival of tumor cells, since a number of plant substances and closely related chemical entities are potent luciferase inhibitors: Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a powerful inhibitor of firefly luciferase with an IC_{50} of approximately 1–2 μ M (Bakhtiarova et al. 2006; Braeuning and Vetter 2012), as is the structurally related NF κ B inhibitor (E)-2-fluoro-4'-methoxystilbene (Braeuning and Vetter 2012). Certain flavonoids, for example, the kinase inhibitor PD98,059 (2'-amino-3'-methoxyflavone) and the widely used aryl hydrocarbon receptor agonist β -naphthoflavone, also inhibit firefly luciferase at concentrations far below what is routinely used for in vitro assays (Auld et al. 2008; Vaas et al. 2014; Wang 2002).

It is quite difficult to find a paper in the literature where the possibility of luciferase enzyme inhibition has been explicitly taken into account by the authors. This does, however, not necessarily mean that all luciferase reporter assays conducted with cells treated with substances such as resveratrol must have yielded inconclusive results (nor do problems with a luciferase reporter assay invalidate the findings obtained with other experimental approaches which are presented in the same paper). A long time of incubation with resveratrol rather seems to reduce the problem of luciferase-inhibitory effects by resveratrol (Braeuning and Vetter 2012), most likely by conversion of resveratrol to less-active metabolites. This time dependency has only been demonstrated for a few cell lines (Braeuning and Vetter 2012) and will most probably differ substantially between individual lines. However, resveratrol is routinely used in concentrations of up to 100 μ M which does by far exceed the concentrations used for luciferase inhibition in the aforementioned study. It thus often remains uncertain whether a reduction in firefly luciferase reporter activity observed under resveratrol treatment is in fact caused by the reduced transactivation potential of the transcription

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factor of interest or whether it might be caused merely by inhibition of catalytic activity of firefly luciferase.

For some publications, it is possible to indirectly reason the absence of pronounced firefly luciferase inhibition by resveratrol. This applies, for example, if luciferase reporter systems for different signaling pathways were applied under identical conditions with one of the reporters being down, but another one being up-regulated by the compound (Kim et al. 2011), or if the observed down-regulation of reporter activity by resveratrol is antagonized by means of genetic (e.g., mutational inactivation of a certain transcription factor binding site in the genetic region driving the expression of the reporter gene) or pharmacological (e.g., treatment of cells with a modulator of the signaling pathway of interest) interference (Huang et al. 2012; Yu et al. 2013).

Unfortunately, not all publications contain this type of experimental approach. When applying the selection criteria (1) resveratrol incubation ≤ 24 h, (2) reporter activity inhibition ≥ 50 %, (3) no up-regulation of another luciferase reporter under identical conditions, and (4) no detailed mechanistic approach (see the above paragraph), a number of recent papers contain conclusions based on luciferase reporter data which might have been corrupted by luciferase inhibition by resveratrol, since data do not allow to firmly exclude this possibility. For example, Ren et al. (2013) reported down-regulation of NF κ B-driven firefly luciferase reporter activity by resveratrol, while the substance did not affect transcription factor abundance, nuclear translocation, and DNA binding affinity. The conclusion of NF κ B inhibition by resveratrol in the paper is solely based on diminished luciferase reporter activity in the presence of resveratrol, the latter finding possibly being due to direct reporter enzyme inhibition rather than interference with the NF κ B transcription factor. Similarly, Shi et al. (2009) showed a reduction in PSA-driven reporter activity by resveratrol, which was observed in the absence of effects on androgen receptor abundance. Kim et al. (2010) tried to establish a link between diminished food intake of resveratrol-exposed mice and reduced expression of the orexigenic neuropeptides NPY and AgRP in vitro, based on the reduction in NPY- and AgRP-driven firefly luciferase reporter activities by resveratrol as sole experimental evidence, which, however, is questionable since the system might have been disturbed by inhibition of firefly luciferase.

There are different ways of avoiding the problems arising when combining luciferase-inhibiting substances such as resveratrol or certain flavonoids with reporter assays. Proper controls, mechanistic approaches, timing, and

choice of substance concentration will reduce the risk of unintended disturbance. Optimized firefly luciferase variants are available which are resistant against many known enzyme inhibitors (Auld et al. 2009). First of all, however, it is crucial to increase awareness for this long-ignored issue.

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