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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of a ligand–quencher conjugate for the ligand binding study of the aryl hydrocarbon receptor using a FRET assay

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Abstract The aryl hydrocarbon receptor (AhR) is a transcription factor that induces the adaptive responses upon binding to a wide range of exogenous chemicals in cells. The traditional method for studying AhR ligands is to determine their effect on transcriptional activities (e.g., luciferase expression) in cultured cells. In this paper, we sought to investigate the feasibility of studying the ligand binding with the AhR using a Förster resonance energy transfer (FRET) assay—a novel approach. Two conjugates (βNFQ and **8**) containing β-naphthoflavone and DABCYL (a quencher) with different linkers were therefore synthesized for evaluation. The luciferase expression and green fluorescent protein (GFP) expression assays showed that βNFQ is a partial AhR agonist. Simultaneous incubation of cultured cells containing expressed GFP-AhR fusion protein with βNFQ and a known AhR ligand 3-methylchloranthrene reduced the fluorescence of GFP-AhR to a lesser extent, suggesting that the reduction in fluorescence resulted from the competitive binding of ligands to GFP-AhR. Although the fluorescent quenching by βNFQ is modest, our results suggest that FRET could be a valuable tool for identifying AhR ligands with the use of a more efficient ligand–quencher.

Keywords Aryl hydrocarbon receptor ligands · Förster resonance energy transfer · β-Naphthoflavone · Green fluorescence protein

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which belongs to the basic helix–loop–helix Per–Arnt–Sim (PAS) protein family. At the resting state, the AhR resides in the cytoplasm in a complex containing a dimer of Hsp90, a molecule of p23, and a molecule of XAP2 (Beischlag *et al.*, 2008). Upon ligand binding to the AhR, a conformational change occurs to the AhR complex, allowing nuclear localization via interaction with the importin protein at the nuclear pore (Petrulis *et al.*, 2003). After translocation into the nucleus, the AhR heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) to form an active transcription factor. The AhR/Arnt heterodimer binds to its corresponding enhancer, leading to the activation of xenobiotic metabolizing gene transcription, most notably cytochrome P450 1A1, 1A2, and 1B1. In addition to this classical AhR function, the AhR has been shown to crosstalk with a number of distinct signaling pathways that involve estrogen receptor (Brunnberg *et al.*, 2003), hypoxia inducible factor-1α (Chan *et al.*, 1999), NFκB (Chan *et al.*, 1999), and cell cycle control (Marlowe *et al.*, 2004). Although the AhR is involved in liver development, immune response,

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and cancer, its endogenous ligand(s) has not been definitively identified (Nguyen and Bradfield, 2008).

Dioxins and polycyclic aromatic hydrocarbons (PAH) are well-known environmental pollutants that bind the AhR. Many of them are suspected carcinogens and most, if not all, of their toxicities are primarily mediated through the AhR (Bock and Kohle, 2006; Mimura and Fujii-Kuriyama, 2003; Nebert and Karp, 2008). Because these environmental agents bind the AhR, it is important to identify the presence of AhR ligands in our environment and to determine the level of human exposure to these agents. The traditional method for studying the ligand–AhR interactions is to measure transcriptional activities mediated by ligand binding in cultured cells (Adachi *et al.*, 2001; Schaldach *et al.*, 1999, Spink *et al.*, 2003). However, assays based on this method either involve toxic [^{3}H]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or are not suitable for capturing the real-time ligand binding to the AhR. Reporter assays also require longer time since synthesis of luciferase mRNA and protein are involved. Furthermore, a large number of studies reported so far do not utilize the human AhR because binding of a ligand to the human AhR is more difficult to detect in vitro.

In the present work, we initiated an investigation of the feasibility of studying the human AhR ligands via the Förster resonance energy transfer (FRET) (Clegg, 1996). FRET is based on the energy transfer from a donor chromophore to a proximate acceptor chromophore, and has been widely used in bioassays in the past decade (Miyawaki, 1997; Vrabioiu and Mitchison, 2006). The design rationale for our assay using FRET is described as follows (Fig. 1). A ligand–quencher conjugate (AhRL-Q) and a GFP-AhR fusion protein are prepared. AhRL-Q binds to fluorescent GFP-AhR and consequently quenches (decreases) the green fluorescence via FRET because the binding brings the quencher of AhRL-Q close to GFP-AhR. The reduction in fluorescence will be less if another AhR ligand competes with AhRL-Q for binding with GFP-AhR. The change in fluorescence in principle is directly correlated to the binding strength of the competing AhR ligand. To prove the above described approach, we synthesized conjugates *β*NFQ (**7**) and **8** containing *β*-napthoflavone (*β*NF) and 4-(4-dimethylaminophenyl)-diazenylbenzoic acid (DABCYL) as the AhR ligand and the quencher, respectively (Fig. 1). Our results revealed that *β*NFQ could effectively bind to the AhR because it activated the gene transcription of two AhR reporter (GFP and luciferase) genes in cultured cells as determined in separated studies. Addition of *β*NFQ to GFP-AhR in cultured cells reduced the green fluorescence as expected. Simultaneous addition of *β*NFQ along with 3-methylchloranthrene (a known potent AhR ligand) into cultured cells that contain expressed GFP-AhR resulted in less fluorescence quenching as compared to addition of *β*NFQ only. To our knowledge, studying the ligand binding to the AhR using a FRET approach is unprecedented, and the results presented here should provide useful guidance for development of more efficient and practical assays, which could be used for

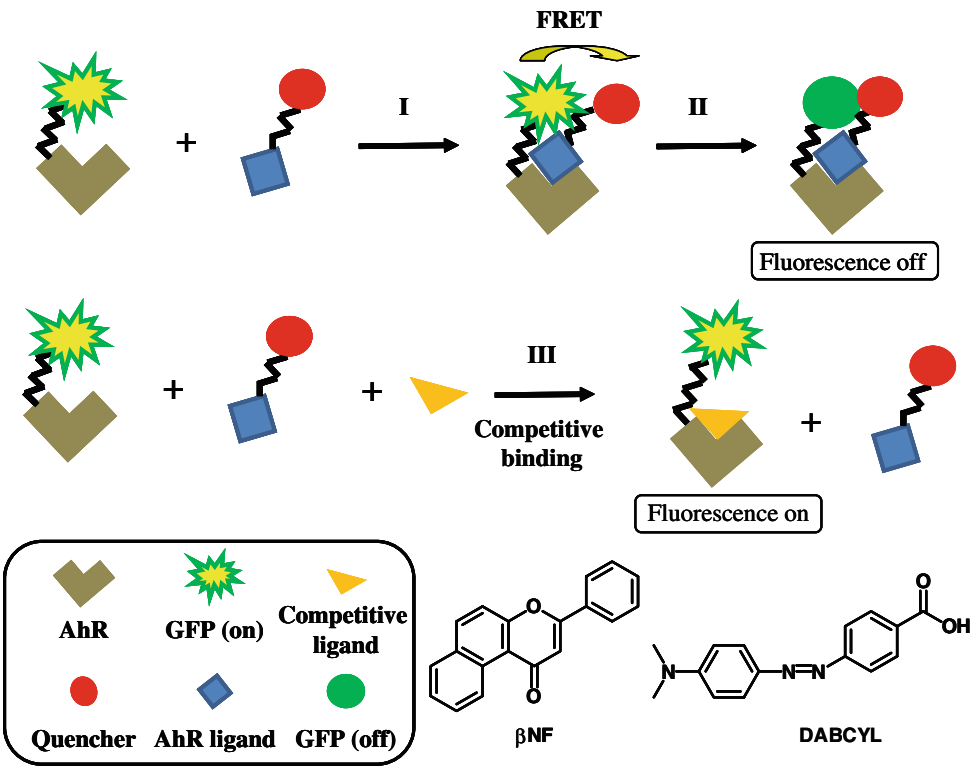


Fig. 1 Graphic demonstration of detecting AhR ligands using FRET. *I* Binding of AhR-Q with GFP-AhR to form a complex, *II* FRET between GFP-AhR and AhR-Q, *III* competitive binding of AhR-Q and another AhR ligand to GFP-AhR. The structures of *β*NF and DABCYL are shown at the *bottom*

studying real-time ligand binding to AhR in living cells and addressing the ligand binding directly whereas reporter assay looks at the down-stream effect after ligand binding.

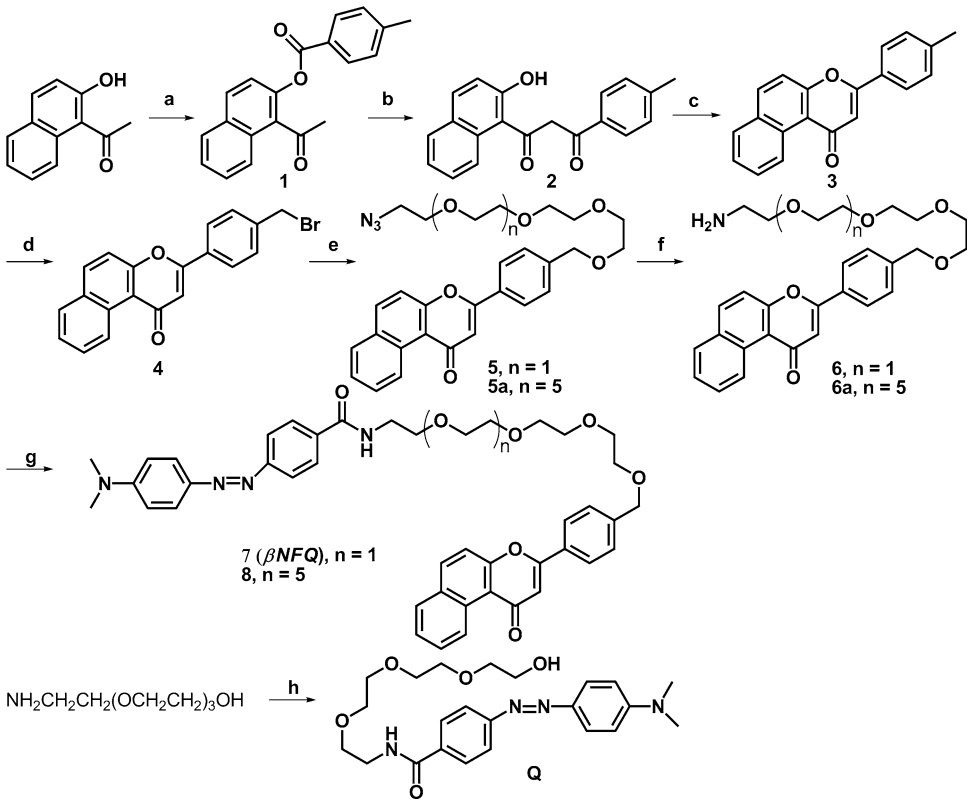
Results and discussion

Chemistry

βNF was chosen for demonstrating our proposed approach because it is a well known AhR ligand with moderate binding affinity (Carver *et al.*, 1994) and is easy to synthesize as a precursor (Ares *et al.*, 1995). Moreover, it has relatively low toxicity to human cells (Godard *et al.*, 2004). DABCYL is a universal dark quencher that is commonly used in the resonance energy transfer assays (Adamczyk *et al.*, 2001; Xie *et al.*, 2009). In our synthesis, the βNF moiety **3** for conjugation was prepared based on the Baker–Venkataraman (B–V) rearrangement as initial steps (Scheme 1). Typically, esterification of 1-acetyl-2-naphthol with *p*-toluoyl chloride followed by treatment of potassium *tert*-butoxide to promote an intramolecular mixed-Claisen rearrangement yielded a diketone **2**. Isolation of **2** was not necessary because a small portion of **2** spontaneously cyclized into **3** (confirmed by ^{1}H NMR, data not shown) during the work-up steps. Acid-catalyzed cyclization of the crude compound **2** produced the βNF moiety **3** in decent yield. Further bromination of the benzylic carbon of **3** using *N*-bromosuccinimide (NBS) followed by reacting with $HO(CH_2CH_2O)_3CH_2CH_2N_3$ and $HO(CH_2CH_2O)_7CH_2CH_2N_3$ yielded **5** and **5a**, respectively. Staudinger reduction of the azide group of **5** and **5a** gave **6** and **6a**. Conjugates **7** (βNFQ) and **8** were prepared by coupling commercially available 4-(4-dimethylaminophenyl)diazenylbenzoic acid succinimidyl ester (DABCYL-SE) with **6** and **6a** (Scheme 1), respectively, followed by column chromatography purification. βNFQ and **8** containing different ethylene glycol linkers were synthesized for determining the effect of the distance between βNF and DABCYL on the FRET efficiency. DABCYL-SE also coupled with $NH_2(CH_2CH_2O)_3CH_2CH_2OH$ to yield Q that was used as a control in our experiments.

βNFQ is a partial AhR agonist

Since we conjugated the quencher molecule to the known AhR ligand βNF, we predicted that both βNFQ and **8** would still bind to the AhR ligand binding domain. It is also possible that the quencher itself might be an AhR ligand because organic extracts from many environmental sources contain AhR ligands, which are known to have diversely distinctive structures (Nguyen and Bradfield, 2008). We examined whether βNFQ and Q activate the DRE-driven GFP expression using the DRE-GFP stable rat hepatoma cell line H4G1.1c3 (Peters *et al.*, 2006). This cell line is stably integrated with the GFP cDNA into the host



Scheme 1 Syntheses of **7** (βNFQ), **8**, and Q. Reagents and conditions: *a* pyridine, *p*-toluyl chloride, r.t., 2 h, 94%; *b* *t*-BuOK, THF, reflux, overnight, 80%; *c* H^+, reflux, 2.5 h, 86%; *d* NBS, AIBN, benzene, reflux, 6 h, 85%; *e* $HO(CH_2CH_2O)_3CH_2CH_2N_3$ (for **5**) or $HO(CH_2CH_2O)_7CH_2CH_2N_3$ (for **5a**), NaH, THF, r.t., 12 h, 63% for **5** and 82% for **5a**; *f* Ph_3P, THF-H_2O, r.t., overnight, 82% for **6** and 79% for **6a**; *g* DABCYL-SE, EDC, TEA, DMF, r.t., 2 h, 75% for **7** and 81% for **8**; *h* DABCYL-SE, EDC, TEA, DMF, r.t., overnight, 83%

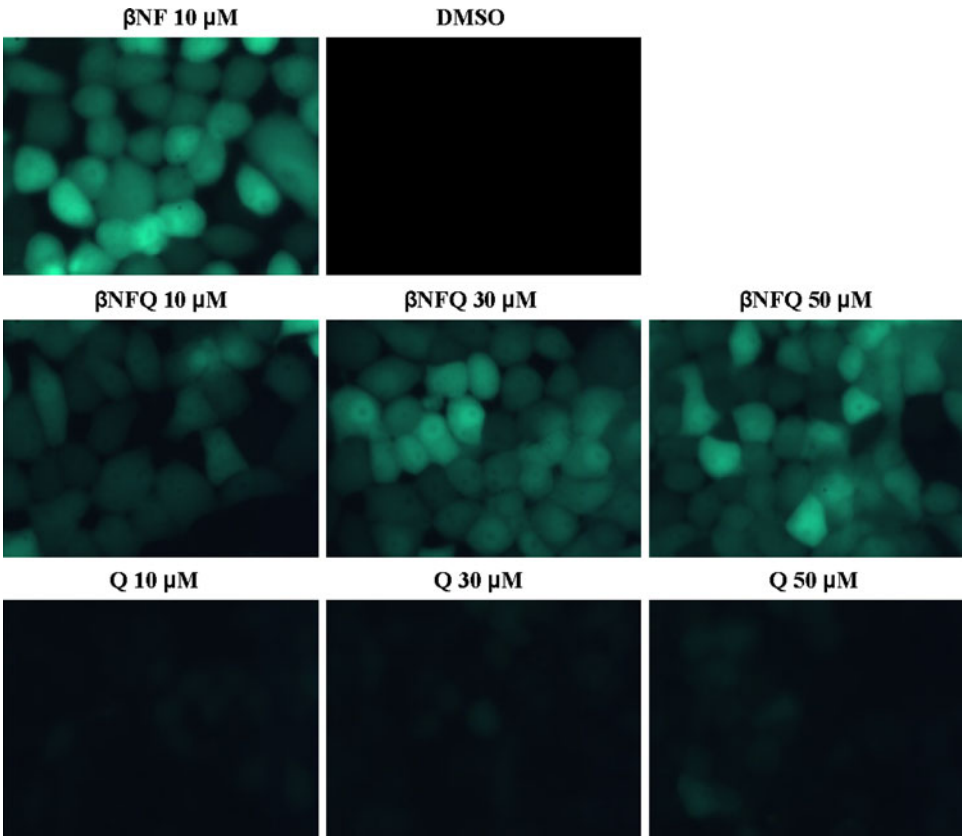


Fig. 2 Fluorescence images showing DRE-driven expression of the GFP in H4G1.1c3 cells. The ligands and their corresponding concentrations are shown

cell genome and the gene transcription of this GFP cDNA is activated after ligand binding to the AhR. A fluorescence microscope was used to measure the green fluorescence emitted from expressed GFP. We observed that 10 μM *β*NF turned on GFP expression after treatment for 48 h whereas DMSO did not cause any GFP expression (Fig. 2). *β*NFQ also activated GFP expression; however, the level of GFP expression was noticeably less intense even at 50 μM concentration. On the other hand, Q appeared to be a relatively weak ligand to the AhR because only some fluorescence was detected at the highest 50 μM concentration (Fig. 2). These results showed that *β*NFQ is a weaker ligand when compared to *β*NF but it can certainly bind to the AhR ligand binding domain much better than Q. In contrast to *β*NFQ, conjugate **8** was cytotoxic to the cells at the same concentrations used for *β*NFQ (Fig. 3); therefore, no GFP expression results could be obtained. Because of this reason, only *β*NFQ was used as a model compound in the following investigations.

In an effort to show whether *β*NFQ is capable of competing with *β*NF for binding, we examined the effect of *β*NFQ on the amount of the *β*NF-driven luciferase protein expression. We chose to quantify the luciferase protein expression rather than performing luciferase assays since *β*NF inhibited the luciferase activity (Wang, 2002). Our quantitative Western data showed that *β*NF (10 μM) induced the production of the luciferase protein by 75-fold (Fig. 4a, b). *β*NFQ (10 μM) significantly induced the luciferase expression by 14-fold whereas Q (10 μM) did not induce luciferase production in any significant amount. Consistent to our prediction, *β*NFQ suppressed the *β*NF induction from 75- to 23-fold and no appreciative difference in *β*NF induction was observed in the presence or absence of Q. These results confirmed the binding of *β*NFQ to the AhR and revealed the binding competition between *β*NFQ and *β*NF, suggesting that *β*NFQ is a suitable model molecule for our designed approach.

*β*NFQ quenches the GFP-AhR fluorescence in a ligand-dependent manner

We expect that the FRET-mediated quenching of *β*NFQ should occur when the quencher molecule is brought physically close to the GFP moiety of GFP-AhR by *β*NF. To test out this hypothesis, we generated Sf9 cells expressing the baculovirus GFP-AhR protein and used them to examine whether *β*NFQ could quench the GFP fluorescence. We realized that the distance between *β*NFQ and GFP is critical for FRET to occur. Therefore, we intentionally fuse GFP to the N-terminus of the full length human AhR protein instead of the C-terminus because GFP would likely be physically closer to the ligand binding

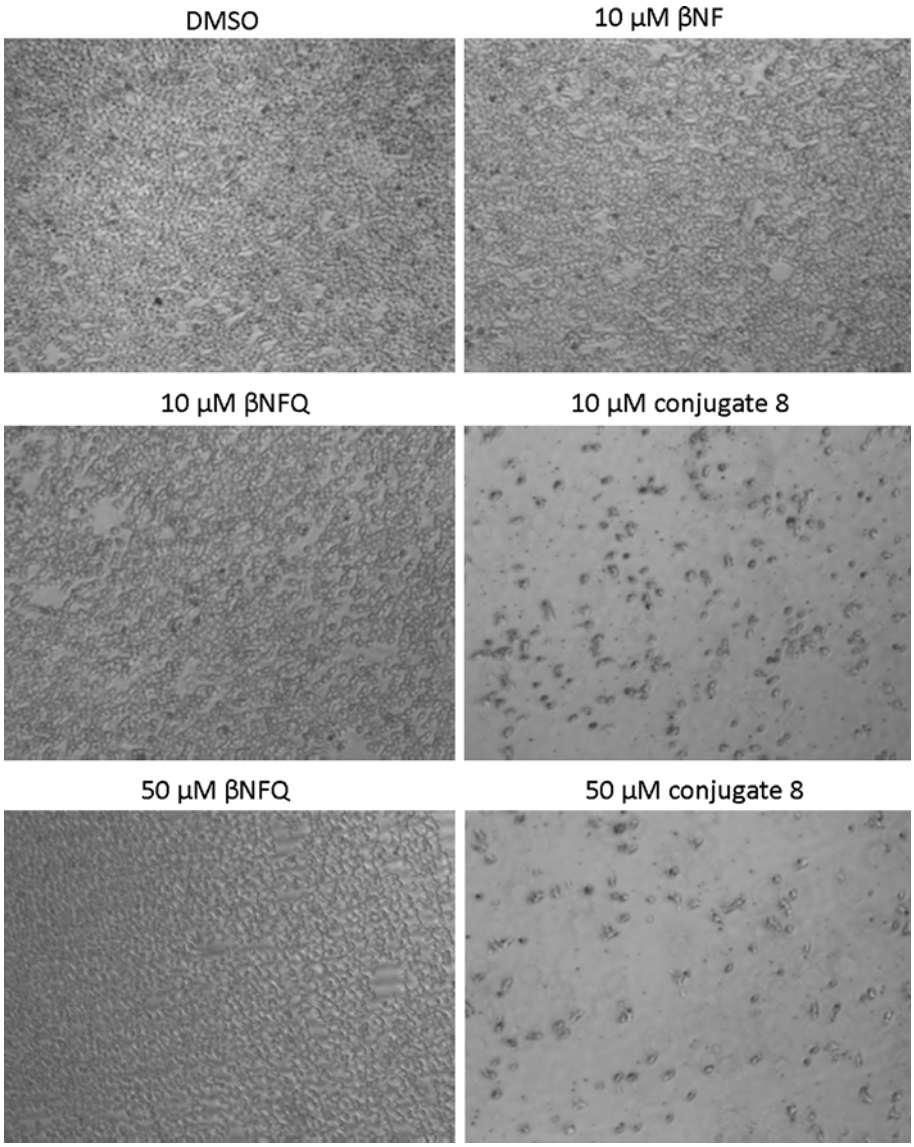


Fig. 3 Images showing cytotoxicity of *β*NF, *β*NFQ, and **8**. The ligands and their corresponding concentrations are shown

domain when GFP is at the N-terminus of the AhR (Fig. 5). When we added *β*NFQ (10 μM) to the GFP-AhR expressing Sf9 cells, we observed 18% quenching as early as 5 min after addition (Fig. 6) and the amount of quenching was similar after 10 min (24%). The quenching appeared to increase only modestly up to 30 min (29% quenching) (data not shown). On the other hand, Q alone also quenched the GFP fluorescence but to a lesser extent: 10 μM Q quenched 13% fluorescence after 5 min and 16% after 10 min. This observed quenching by Q is expected since Q has a weak affinity to the AhR ligand binding domain and the effective Q quenching concentration could conceivably be at micromolar concentrations. Nonetheless, *β*NFQ quenched the GFP fluorescence more effectively than Q in a statistically significant manner. Unfortunately the binding affinity of *β*NF to the AhR is in low micromolar range and the binding affinity of *β*NFQ is considerably weaker than *β*NF, which may explain the weak quenching effect of *β*NFQ at 10 μM concentration. To examine whether an AhR ligand would compete with *β*NFQ for binding to the AhR, we chose to add 3-methylchloranthrene (3MC) simultaneously with *β*NFQ rather than a delayed addition of *β*NFQ to minimize *β*NFQ binding, realizing that AhR ligands in general might have a slow dissociation rate that a stepwise addition of two ligands might reduce the binding competition (Bohonowych and Denision, 2007). Interestingly, 3MC (5 μM), which is a more potent AhR ligand than *β*NF, reversed the *β*NFQ quenching similarly at 5 and 10 min after addition (from 18 to 6.6% at 5 min after addition) in a statistically significant manner whereas no reversal of Q quenching was observed by 3MC (Fig. 6). The reversal of the *β*NFQ quenching by 3MC supported our hypothesis that the quenching effect of *β*NFQ is partly mediated by the

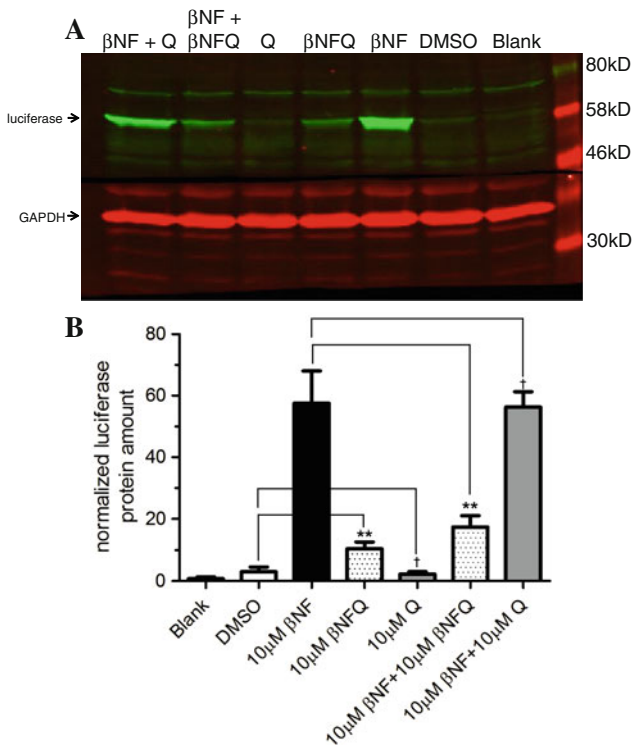


Fig. 4 Quantitative Western blot analysis of DRE-driven luciferase protein expression in Hep3B cells. A representative western data was shown in **a** and the analyzed data were plotted in **b**. *Arrows* indicate luciferase and GAPDH proteins. Luciferase protein amount was normalized by the GAPDH content. The *error bar* represents ± SD ($n = 3$). $^{**}P < 0.009$ (significant); † $P > 0.4$ (not significant)

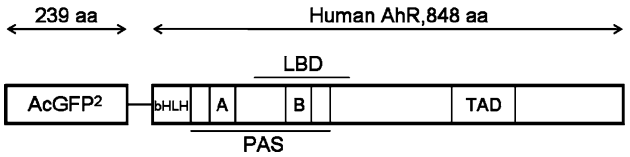


Fig. 5 Domain map of the GFP-AhR protein. GFP (239 amino acids) is fused to the N-terminus of the full length human AhR protein (848 amino acids). AcGFP^2, GFP; bHLH, basic helix–loop–helix DNA binding domain; PAS, signature domain for bHLH-PAS protein family which contains PAS A and PAS B; LBD, ligand binding domain; TAD, transactivation domain responsible for recruitment of coactivators which facilitate activation of gene transcription

binding of *β*NFQ to AhR since the presence of 3MC, which likely displaced *β*NFQ from the ligand binding domain, reduced the quenching effect. We realized that the amount of quenching is modest, and *β*NFQ may not be an ideal molecule for a practical FRET assay as proposed above. However, our results clearly suggest that use of FRET to identify AhR ligands is definitely conceivable and warrants further investigation. In addition, it should be emphasized that the quenching efficiency is governed by the ligand binding to the human AhR. At present, most of the AhR ligand binding studies have been performed using a non-human AhR which binds ligand well. However, it is more difficult to study ligand binding using the human AhR. The fact that we were able to use our current set up to study ligand binding using the human AhR is encouraging; our study provides us a more realistic measure of how a ligand binds AhR in humans.

Conclusions

Here we have proved that when we conjugated a quencher molecule to *β*NF, *β*NF has affinity to the AhR ligand binding domain and increases the quenching effectiveness by probably increasing the quencher concentration at the GFP vicinity. Although the quenching activity of *β*NFQ is very modest, it proves that our hypothesis of designing an AhR ligand–quencher molecule to study human AhR ligand binding via FRET is certainly viable. From our data, we predict that it would be more effective to quench the GFP fluorescence if the quencher is conjugated with an AhR ligand (e.g., 3MC) of low micromolar or nanomolar binding affinity. Replacement of DABCYL with a stronger quencher could be another choice to improve the fluorescence quenching efficiency. The work presented here is the first effort to develop a potentially novel approach for studying AhR ligands via FRET. We believe that the FRET assay could be complementary to the traditional "DRE-driven luciferase expression" assay. This approach may enable us to ultimately generate quencher reagents using GFP-AhR to study ligand binding to the human AhR in real-time and to screen for potential AhR ligands from various sources in a high-throughput manner. Synthesis of conjugates containing stronger AhR ligands and more efficient quenchers are currently ongoing in our laboratory and the evaluation results will be presented in due course.

Experimental section

Materials and methods

Chemicals for synthesis were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification. Reactions were carried out under Argon using dry solvent, unless otherwise noted. IR spectra were collected on a Shimadzu (Pleasanton, CA) IR Prestige-21 FT-IR spectrophotometer. ^{1}H and ^{13}C spectra were collected on a JEOL (Peabody, MA) ECA 600 MHz FT-NMR spectrometer. HRMS (DART-TOF) spectra were collected on a JEOL (Peabody, MA) Accu-TOF LC^{TM} time-of-flight mass spectrometer equipped with an open-air ion source Direct Analysis in Real Time (DART). Fluorescence was measured on an Odyssey LI-COR (Lincoln, NE) infrared

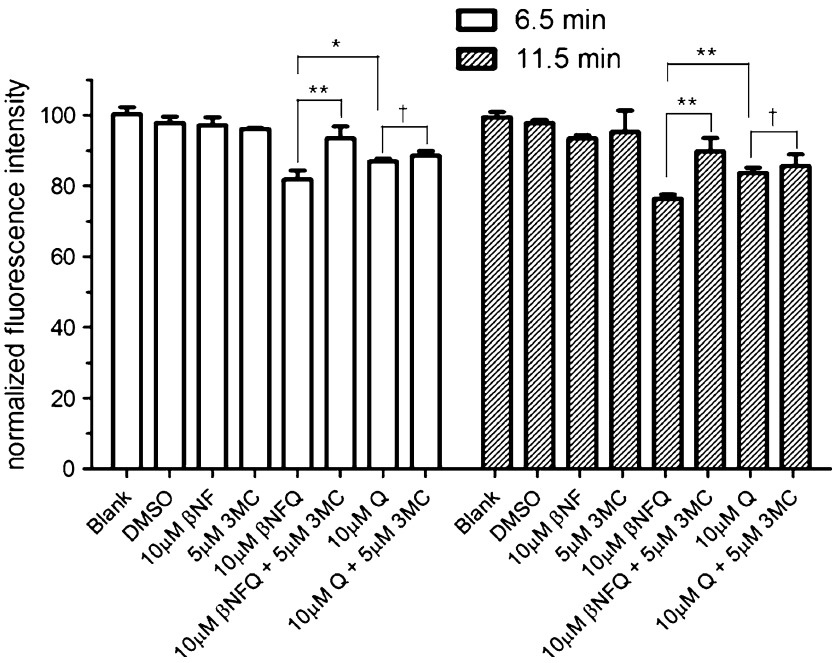


Fig. 6 Fluorescence quenching studies of βNFQ using GFP-AhR expressing Sf9 cells. The *error bar* represents ± SD of three independent experiments ($n = 3$). $^{*}P < 0.03$ (significant); $^{**}P < 0.01$ (very significant); † $P > 0.14$ (not significant)

imaging system. Fluorescence images were collected on a Nikon (Melville, NY) Eclipse TE200 fluorescence microscope. Dulbecco's modified Eagles medium (DMEM) was purchased from Sigma (St. Louis, MO). Advanced MEM, glutaMAX, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Tissue Culture Biologicals (Seal Beach, CA). β-Naphthoflavone (βNF) and 3-methylcholanthrene (3MC) were purchased from Sigma (St. Louis, MO).

Preparation of **1**

To a pyridine solution (40 ml) of 1-acetyl-2-naphthol (2 g, 10.7 mmol) at 0°C, was added *p*-toluyl chloride (1.72 ml, 12.9 mmol). After stirring at room temperature for 2 h, the reaction was quenched with a mixture of 5% HCl/crushed ice (1:1, 40 ml). The precipitate was collected and dissolved in ethyl acetate (100 ml). The organic solvent was washed with brine (20 ml), dried over anhydrous Na_2SO_4, and concentrated. Flash chromatography of the residue (silica gel, EtOAc:hexane; 1:15) yielded **1** (3.07 g, 94.2%) as a white solid. IR (KBr): 1740, 1704, 1261, 1207, 1177, 1068, 746 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.09 (d, 2H, $J = 8.28$ Hz), 7.94 (d, 1H, $J = 8.94$ Hz), 7.90 (d, 1H, $J = 8.22$ Hz), 7.83 (d, 1H, $J = 8.22$ Hz), 7.54 (m, 2H), 7.38 (d, 1H, $J = 8.88$ Hz), 7.33 (d, 2H, $J = 8.28$ Hz), 2.61 (s, 3H), 2.46 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 203.0, 164.9, 145.1, 131.6, 131.0, 130.4, 129.9, 129.5, 128.4, 127.7, 126.2, 126.0, 124.5, 121.5, 32.5, 21.8; HRMS (DART-TOF) calcd for $C_{20}H_{17}O_3$ ($M + H^+$) 305.1178, found 305.1160.

Preparation of **2**

To a THF solution (20 ml) of **1** (500 mg, 1.6 mmol), was added potassium *tert*-butoxide (202 mg, 1.8 mmol). After refluxing overnight, the reaction mixture was cooled to room temperature and adjusted to pH 3 by HCl (3 M). The reaction mixture was extracted with dichloromethane (50 ml), and the organic layer was washed with 5% $NaHCO_3$ (50 ml × 2), brine (50 ml × 2), and water (50 ml), dried over anhydrous Na_2SO_4, and concentrated to yield **2** (400 mg, 80%) as a pale yellow solid. HRMS (DART) calcd for $C_{20}H_{17}O_3$ ($M + H^+$) 305.1178, found 305.1155. This product was used for next step without further purification.

Preparation of **3**

To an acetic acid solution (17 ml) of **2** (400 mg, 1.3 mmol), was added concentrated H_2SO_4 (0.87 ml, 16 mmol). After refluxing for 2.5 h, the reaction mixture was quenched with a mixture of crushed ice (100 ml) and water (35 ml). The mixture was extracted with CH_2Cl_2 (100 ml), and the organic layer was washed with brine (50 ml × 2), dried over anhydrous Na_2SO_4, and concentrated. Flash chromatography of the residue (silica gel, CH_2Cl_2) yielded **3** (325 mg, 86.2%) as a pale yellow solid. IR (KBr): 2356, 1636, 1509, 1269, 817, 752 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 10.10 (d, 1H, $J = 8.22$ Hz), 8.12 (d, 1H, $J = 8.94$ Hz), 7.92 (d, 1H, $J = 7.56$ Hz), 7.87 (d, 2H, $J = 8.28$ Hz), 7.77 (m, 1H), 7.63 (m, 2H), 7.35 (d, 2H, $J = 8.51$ Hz), 6.96 (s, 1H), 2.46 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 180.5, 161.1, 157.4, 142.1, 135.4, 129.8, 129.2,

128.2, 127.2, 126.7, 126.1, 117.7, 109.9, 21.6; HRMS (DART-TOF) calcd for $C_{20}H_{15}O_2$ ($M + H^+$) 287.1072, found 287.1078.

Preparation of **4**

To a hot benzene solution (16 ml) of **3** (200 mg, 0.7 mmol), was added *N*-bromosuccinimide (160 mg, 0.90 mmol) and azobisisobutyronitrile (60 mg, 0.37 mmol). After refluxing for 6 h, the reaction mixture was cooled to room temperature, and the precipitate was collected by filtration and washed by benzene (10 ml × 2) to yield **4** (218 mg, 85.3%) as a yellow solid. IR (KBr): 3076, 1932, 1771, 1639, 1601, 1511, 1234, 957, 835, 645, 593 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.08 (d, 1H, $J = 8.94$ Hz), 8.13 (d, 1H, $J = 8.94$ Hz), 7.94 (d, 2H, $J = 8.36$ Hz), 7.92 (d, 1H, $J = 7.76$ Hz), 7.78 (t, 1H, $J = 7.70$ Hz), 7.64 (m, 2H), 7.57 (d, 2H, $J = 8.36$ Hz), 6.99 (s, 1H), 4.55 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 160.2, 135.7, 131.5, 130.7, 130.5, 129.8, 129.4, 128.3, 127.3, 126.8, 126.6, 117.6, 110.8, 32.4, 29.7; HRMS (DART-TOF) calcd for $C_{20}H_{14}BrO_2$ ($M + H^+$) 365.0177, found 365.0179.

Preparation of **5**

To a THF solution (anhydrous, 10 ml) of $HO(CH_2CH_2O)_2CH_2CH_2N_3$ (200 mg, 0.91 mmol), was added NaH (11 mg, 31.7 mg of 60% NaH in mineral oil, 0.46 mmol), and the reaction mixture was allowed to stir for 10 min. To the reaction mixture was then added **4** (300 mg, 0.82 mmol). After stirring for 12 h at room temperature, the reaction mixture was quenched by adding CH_3OH, filtered on Celite 545, and concentrated. Flash chromatography of the residue (silica gel, $MeOH/CH_2Cl_2$; 1:70) yielded **5** (260 mg, 63%) as a yellow solid. IR (KBr): 2861, 2099, 1636, 1508, 1439, 1390, 1341, 1285, 1243, 1098, 957, 820, 754 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.03 (d, 1H, $J = 8.22$ Hz), 8.03 (d, 1H, $J = 8.03$ Hz), 7.87 (d, 2H, $J = 8.22$ Hz), 7.84 (d, 1H, $J = 8.22$ Hz), 7.71 (t, 1H, $J = 8.58$ Hz), 7.56 (t, 1H, $J = 6.86$ Hz), 7.52 (d, 1H, $J = 8.89$ Hz), 7.47 (d, 2H, $J = 8.22$ Hz), 6.90 (s, 1H), 4.61 (s, 2H), 3.67 (m, 14H), 3.37 (t, 2H, $J = 5.46$ Hz); ^{13}C NMR ($CDCl_3$) δ 180.2, 160.6, 157.3, 142.2, 135.4, 130.6, 130.5, 129.2, 128.2, 128.0, 127.2, 126.6, 126.1, 117.6, 117.2, 110.3, 72.6, 70.8, 70.1, 69.9, 50.7, 24.8; HRMS (DART-TOF) calcd for $C_{28}H_{30}N_3O_6$ ($M + H^+$) 504.2135, found 504.2134.

Preparation of **6**

To a THF solution (5 ml) of **5** (54 mg, 0.11 mmol) at room temperature, was added triphenylphosphine (39 mg, 0.15 mmol), and the reaction mixture was allowed to stir for 2 h. To the reaction mixture, was added H_2O (0.5 ml), and the reaction mixture was stirred overnight and concentrated. Flash chromatography of the residue (silica gel, CH_2Cl_2:MeOH:Et_3N; 100:10:1) yielded **6** (42 mg, 82%) as a yellow solid. IR (KBr): 3429, 2103, 1635, 1506, 1439, 1352, 1100, 823 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.01 (d, 1H, $J = 8.58$ Hz), 8.03 (d, 1H, $J = 8.94$ Hz), 7.87 (d, 2H, $J = 8.22$ Hz), 7.84 (d, 1H, $J = 7.92$ Hz), 7.69 (m, 1H), 7.54 (m, 2H), 7.46 (d, 2H, $J = 8.58$ Hz), 6.90 (s, 1H), 4.59 (s, 2H), 3.65 (m, 14H), 3.48 (t, 2H, $J = 5.16$ Hz), 2.82 (t, 2H, $J = 5.16$ Hz); ^{13}C NMR ($CDCl_3$) δ 180.1, 160.5, 157.2, 141.9, 135.3, 130.43, 130.36, 130.3, 129.0, 128.0, 127.9, 127.0, 126.4, 126.0, 117.4, 117.0, 110.1, 72.4, 72.6, 70.50, 70.45, 70.42, 70.1, 69.7, 41.4); HRMS (DART) calcd for $C_{28}H_{32}NO_6$ ($M + H^+$) 478.2230, found 478.2262.

Preparation of **7** (βNFQ)

To a DMF solution (10 ml) of DABCYL-SE (30 mg, 0.11 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 23 mg, 0.12 mmol), 1-hydroxy-benzotriazole (HOBt, 16.5 mg, 0.12 mmol), was added **6** (30 mg, 0.088 mmol). After stirring at room temperature for 2 h, the organic solvent was removed under vacuum. Flash chromatography of the residue (silica gel, CH_2Cl_2:MeOH; 100:3) yielded **7** (48 mg, 74.9%) as a red solid. IR (KBr): 2955, 2914, 2869, 1634, 1599, 1514, 1422, 1366, 1136, 819 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.07 (d, 1H, $J = 8.94$ Hz), 8.07 (d, 1H, $J = 8.22$ Hz), 7.95 (d, 2H, $J = 8.22$ Hz), 7.89 (d, 1H, $J = 7.56$ Hz), 7.82 (m, 6H), 7.75 (m, 1H), 7.61 (t, 1H, $J = 8.22$ Hz), 7.56 (d, 1H, $J = 8.94$ Hz), 7.42 (d, 2H, $J = 8.28$ Hz), 6.89 (s, 1H), 6.62 (d, 2H, $J = 8.88$ Hz), 4.53 (s, 2H), 3.67 (m, 14H), 3.62 (m, 1H), 3.47 (s, 1H), 2.95 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 180.3, 167.2, 160.6, 157.3, 154.9, 152.6, 143.5, 141.9, 135.4, 134.7, 130.6, 130.51, 130.45, 129.2, 128.2, 128.1, 128.0, 127.6, 126.1, 125.3, 122.1, 117.7, 117.2, 111.3, 110.2, 72.5, 70.61, 70.57, 70.2, 70.0, 69.7, 40.1, 40.0; HRMS (DART) calcd for $C_{43}H_{45}N_4O_7$($M + H^+$) 729.3288, found 729.3334.

Preparation of **5a**

To a THF solution (anhydrous, 10 ml) of $HO(CH_2CH_2O)_7CH_2CH_2N_3$ (220 mg, 0.557 mmol), was added NaH (14 mg, 23 mg of 60% NaH in mineral oil, 0.6 mmol), and the reaction mixture was allowed to stir for 10 min. To the reaction mixture was then added **4** (220 mg, 0.6 mmol). After stirring for 12 h at room temperature, the reaction mixture was quenched by adding CH_3OH, filtered on Celite 545, and concentrated. Flash chromatography of the residue (silica gel, $MeOH/CH_2Cl_2$; 1:40) yielded **5a** (310 mg, 82%) as a yellow oil. IR (KBr): 2868, 2111, 1638, 1610, 1512, 1439, 1349, 1293, 1244, 1109, 963, 823 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.07 (d, 1H, $J = 8.22$ Hz), 8.12 (d, 1H,

$J = 9.11$ Hz), 7. 95 (d, 2H, $J = 8.06$ Hz), 7.92 (d, 1H, $J = 7.81$ Hz), 7.77 (t, 1H, $J = 6.94$ Hz), 7.64 (m, 2H), 7.52 (d, 2H, $J = 7.81$ Hz), 7.02 (s, 1H), 4.66 (s, 2H), 3.63-3.71 (m, 30H), 3.37 (t, 2H, $J = 4.93$ Hz); ^{13}C NMR ($CDCl_3$) δ 180.4, 160.9, 157.5, 142.3, 135.7, 130.7, 130.5, 129.4, 128.3, 128.1, 127.3, 126.8, 126.3, 117.7, 117.3, 110.3, 72.7, 70.8, 70.7, 70.1, 50.8; HRMS (DART-TOF) calcd for $C_{36}H_{46}N_3O_{10}$ ($M + H^+$) 680.3138, found 680.3246.

Preparation of **6a**

To a THF solution (10 ml) of **5a** (290 mg, 0.427 mmol) at room temperature, was added triphenylphosphine (180 mg, 0.687 mmol), and the reaction mixture was allowed to stir for 2 h. To the reaction mixture, was added H_2O (1 ml), and the reaction mixture was stirred overnight and concentrated. Flash chromatography of the residue (silica gel, CH_2Cl_2:MeOH:Et_3N; 100:10:1) yielded **6a** (220 mg, 79%) as a yellow oil. IR (KBr): 3465, 2875, 1641, 1596, 1516, 1439, 1352, 1290, 1241, 1105, 963, 827 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 10.06 (d, 1H, $J = 9.03$ Hz), 8.10 (d, 1H, $J = 8.13$ Hz), 7.93 (d, 2H, $J = 8.22$ Hz), 7.90 (d, 1H, $J = 7.82$ Hz), 7.74 (t, 1H, $J = 7.83$ Hz), 7.61 (m, 2H), 7.51 (d, 2H, $J = 8.31$ Hz), 6.95 (s, 1H), 4.64 (s, 2H), 3.64–3.69 (m, 30H), 3.60 (t, 2H, $J = 5.18$ Hz); ^{13}C NMR ($CDCl_3$) δ 180.3, 160.8, 157.5, 142.2, 135.5, 130.6, 129.3, 128.3, 128.1, 127.2, 126.7, 126.2, 117.7, 117.3, 110.4, 72.6, 71.5, 70.7, 70.6, 70.5, 70.4, 70.3, 70.2, 69.9, 41.4; HRMS (DART) calcd for $C_{36}H_{48}NO_{10}$ ($M + H^+$) 654.3280, found 654.3210.

Preparation of **8**

To a DMF solution (20 ml) of DABCYL-SE (89 mg, 0.33 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 67 mg, 0.35 mmol), 1-hydroxy-benzotriazole (HOBt, 48 mg, 0.35 mmol), was added **6** (200 mg, 0.306 mmol). After stirring at room temperature for 2 h, the organic solvent was removed under vacuum. Flash chromatography of the residue (silica gel, CH_2Cl_2:MeOH; 100:3) yielded **8** (225 mg, 81%) as a red solid. IR (KBr): 2868, 1638, 1599, 1516, 1443, 1363, 1293, 1244, 1140, 963, 823 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 10.07 (d, 1H, $J = 8.66$ Hz), 8.10 (d, 1H, $J = 8.95$ Hz), 7.94–7.85 (m, 9H), 7.75 (t, 1H, $J = 7.01$ Hz), 7.61 (m, 2H), 7.49 (d, 2H, $J = 8.07$ Hz), 6.89 (s, 1H), 6.73 (d, 2H, $J = 8.77$ Hz), 4.62 (s, 2H), 3.68-3.59 (m, 32H), 3.07 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 207.1, 180.4, 167.1, 160.8, 157.4, 142.2, 135.5, 134.8, 130.7, 130.6, 129.3, 128.3, 128.1, 127.3, 126.7, 126.2, 122.1, 117.7, 117.3, 111.7, 110.4, 72.6, 70.7, 70.6, 70.6, 70.3, 69.9, 53.5, 40.4, 40.0, 31.0; HRMS (DART) calcd for $C_{51}H_{61}N_4O_{11}$($M + H^+$) 905.4337, found 905.4432.

Preparation of Q

To a DMF solution (5 ml) of $HO(CH_2CH_2O)_3CH_2CH_2NH_2$ (30 mg, 0.16 mmol), was added DABCYL-SE (30 mg, 0.082 mmol). After stirring at room temperature overnight under Ar, the reaction mixture was concentrated under vacuum. Flash chromatography of the residue (silica gel, CH_2Cl_2:MeOH; 100:3) yielded Q (30 mg, 83%) as an orange-yellow solid. IR (KBr): 3465, 3340, 2916, 2861, 2340, 1718, 1652, 1617, 1554, 1154, 1077, 945, 892, 830 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.96 (d, 2H, $J = 8.3$ Hz), 7.87 (d, 2H, $J = 9.6$ Hz), 7.836 (d, 2H, $J = 8.3$ Hz), 6.73 (d, 2H, $J = 8.9$ Hz), 3.59-3.68 (m, 16H), 3.07 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 167.3, 154.8, 152.7, 143.5, 134.9, 128.2, 125.2, 121.9, 111.4, 72.4, 70.5, 70.4, 70.3, 70.0, 69.7, 61.3, 40.2, 39.8; HRMS (DART-TOF) calcd for $C_{23}H_{33}N_4O_5$ ($M + H^+$) 445.2451, found 445.2461.

Ligand binding study using DRE-GFP stable cell line H4G1.1c3

H4G1.1c3 cells that contain a stably integrated enhanced GFP cDNA controlled by a cytochrome P450 1A1 promoter fragment were engineered from a mouse hepatoma cell line Hepa1c1c7 (Peters *et al.*, 2006). H4G1.1c3 cells were maintained in DMEM medium supplemented with 10% FBS, L-glutaMAX (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Half of a milliliter of cell suspension (5×10^5 cells per ml of fresh media) was seeded in each well of a 24-well cell culture plate and the cells were incubated in 5% CO_2 at 37°C for 24 h. The cells were then treated with the molecule of interest (*β*NF, *β*NFQ, Q or vehicle DMSO) and further incubated in 5% CO_2 at 37°C for 48 h. Media was then aspirated by vacuum and PBS (500 μl) was added to rinse the cells by gently rocking the plate for a few seconds before aspiration. This PBS wash was repeated two more times to ensure removal of all the media content. Finally cells were kept in PBS (500 μl) for fluorescence analysis (Ex: 465–495 nm; Em: 515–555 nm; exposure time: 5.56 s; gain: 7.1758).

Cytotoxicity study of *β*NF, *β*NFQ, and **8**

H4G1.1C3 cell suspension (1.2×10^5 cells, 1 ml of media) was seeded into each well of a 12-well. Cells were incubated at 37°C, 5% CO_2 overnight. Then cells were treated with different molecules (DMSO, *β*NF, *β*NFQ, and **8**) at different concentration for 24 h at 37°C, 5% CO_2. After treatment, cells were washed with 1 ml of ice-chilled PBS for three times. The washed cells were photographed in PBS (1 ml) under microscope. The camera was set to exposure time at 4.12 ms and gain at 1.

Quantitative western blot analysis showing DRE-driven luciferase expression

Hep3B cells were seeded into two 24-well plates with 1.25×10^5 cells per well in growth media (advanced MEM containing 5% charcoal stripped FBS, 2 mM L-glutaMAX, 100 U/ml penicillin, 100 μg/ml streptomycin) and incubated at 37°C, 5% CO_2 for 24 h. pGudLuc1.1 (200 ng) was transfected into each well using Fugene HD (Roche) with the ratio of Fugene HD to DNA at 2 μl:1 μg in serum starvation media (advanced MEM containing 3% charcoal stripped FBS, 2 mM L-glutaMAX), and incubated at 37°C, 5% CO_2 for 6 h. Afterwards media was discarded and 500 μl of growth media was added to each well. Cells were treated by a compound, followed by incubation at 37°C, 5% CO_2 for 6 h before harvest. Each treatment condition involved two wells of cells. After washing with ice-chilled PBS for three times, cells with the same treatment were harvested into 50 μl of 1× SDS-PAGE sample buffer (25 μl per well). Each sample was sonicated on ice for 1 s, followed by separation on a 12% SDS-PAGE gel using a BIO-RAD mini-PROTEAN 3 electrophoresis system. The gel was then transferred onto a nitrocellulose membrane (Millipore) in wet transfer buffer at 200 mA for 90 min using a Bio-RAD mini Trans-Blot system. After incubating for 1 h at room temperature in blocking buffer (LI-COR) with gently shaking, the transferred membrane was incubated in blocking buffer containing anti-luciferase antibody (Promega, 1:1,000 dilution) and anti-GAPDH antibody (1:10,000 dilution) overnight with gentle shaking. After four times washing with PBS containing 0.1% Tween-20 for 5 min at room temperature, the membrane was incubated in blocking buffer containing Odyssey donkey anti-goat IRDye 800CW (Cat #827-08952, 1:10,000 dilution) and goat anti-rabbit IRDye 680 (Cat #82708367, 1:10,000 dilution) for 1 h at room temperature in the dark with gentle shaking. Afterwards membrane was washed in PBS containing 0.1% Tween-20 for 5 min at room temperature for four times and a final wash with PBS alone for 5 min. The washed membrane was then dried on a filter paper, ready for analysis using an Odyssey infrared imaging system (LI-COR). All treatments were performed in triplicate. The amount of the luciferase protein expressed was normalized by the GAPDH content in each sample.

Baculovirus GFP-AhR expression in Sf9 cells

The full length human AhR cDNA was cloned into the *Xho*I and *Sac*II sites of the pAcGFP^2-C1 plasmid (Clontech) to generate the pAcGFP^2-C1-AhR plasmid. Then the GFP-AhR cDNA was amplified by PCR and cloned into the *Pst*I/*Not*I sites of the pVL1392 baculovirus transfer plasmid (Pharmingen) to generate the final pVL1392-GFP-AhR plasmid for baculovirus production. The GFP-AhR cDNA was sequenced to confirm the identity. BaculoGold kit (Pharmingen) was used to generate GFP-AhR cDNA containing baculovirus, according to the manufacturer's recommendation. Routinely we infected Sf9 cells ($\sim 6 \times 10^4$ cells) in a well of a 96-well plate with 5 μl of the high titer GFP-AhR baculoviral stock. Two days after infection, expression of the fluorescent GFP-AhR protein can be observed in live cells under a fluorescent microscope.

Fluorescence quenching assay

Sf9 cells were grown in Gibco SF-900 SFM media (Invitrogen) in a 96-well plate. Each well contained 100 μl of media with 80% confluent Sf9 cells before infection with baculovirus. The high titer baculoviral stock (about MOI of 10) contained the GFP-AhR expressing baculovirus. Five microliter of this stock was added to each well to infect the Sf9 cells. Fluorescence was monitored 2 days after infection. Cells were washed three times by PBS and kept in 100 μl of PBS per well before the 96-well plate was focused using a Nikon Eclipse TE200 fluorescence microscope. The lamp was switched under the GFP fluorescence mode and the camera was set to exposure time at 8.57 s and gain at 15.3203. Then set up the timer before adding compounds and shaking the plate, and put the plate back under the microscope exactly at 30 s time point. The camera was allowed to focus for 1 min and then take the first picture. Without changing the field, pictures were subsequently taken after 5 and 10 min. Image J software was used to determine the amount of fluorescence of the whole picture.

Statistical analysis

GraphPad Prism 5 software was used to determine the statistical significance. Two-tailed unpaired *t*-test was performed to show statistically significant ($P < 0.05$) and insignificant ($P > 0.05$) data.

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