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A Novel Fluorescence-Based Assay for the Measurement of Biliverdin Reductase Activity

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ABSTRACT | Biliverdin reductase (BVR) is the enzyme responsible for the last step in the production of bilirubin from the breakdown of heme. Bilirubin is one of the most potent antioxidant molecules in the body. Monitoring BVR activity is essential in studying the antioxidant capacity of cells and tissues. Traditional methods of determining BVR activity have relied on the measurement of bilirubin converted from biliverdin using absorbance spectroscopy. The approach has limited sensitivity and requires large quantities of cells or tissues. We have developed a novel fluorescence-based method utilizing the eel protein, UnaG, for the detection of bilirubin produced by BVR. The UnaG protein only fluoresces by the induction of bilirubin. We have also used this approach to measure intracellular bilirubin content of cultured cells. We validated this assay using cell lysates from mouse liver and immortalized murine hepatic cell line (Hepa1c1c7) and kidney cell line (MCT) in which BVR isoform A (BVRA) was either knocked out via CRISPR or stably overexpressed by lentivirus. Also, we tested the method using previously reported putative BVRA inhibitors, closantel and ebselen. These studies show a new method for measuring bilirubin intracellularly and in lysates.

KEYWORDS | Antioxidant; Bilirubin; Heme oxygenase; Oxidative stress; UnaG

ABBREVIATIONS | BVR, Biliverdin reductase; BVRA, BVR isoform A; HO, heme oxygenase; MCT, mouse cortical tubule; NADP, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PPARα, peroxisome proliferator-activated receptor alpha; SDS, sodium dodecyl sulfate

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1. INTRODUCTION AND METHOD PRINCIPLE

Heme oxygenase (HO) is a critical component of a major antioxidant pathway by the generation of bilirubin [1, 2]. Initially, HO breaks down heme to biliverdin, free iron, and carbon monoxide, and biliverdin is then reduced to bilirubin by the enzyme biliverdin reductase (BVR) [1, 2]. In adults, this reaction is catalyzed by the A isoform of BVR (BVRA). Bilirubin is one of the most potent antioxidant molecules in the body [3, 4], and has been demonstrated to play protective roles against a wide range of diseases and pathologic conditions including ischemia reperfusion-injury, fatty liver disease, obesity, and diabetes [5–12]. Recently, bilirubin has been shown to bind to the peroxisome proliferator-activated receptor alpha (PPARα), which is the only function known outside of its antioxidant properties [6]. PPARα is a transcription factor that reduces adiposity by upregulating genes for β-oxidation. Given these fundamental roles, methods for the accurate measurement of bilirubin production through BVR activity in cells and tissues are necessary for the nuclear receptor, ROS, and antioxidant fields.

Previous methods used for measuring BVR activity or bilirubin content in tissues and cells have been principally based on differential absorbance of bilirubin at specific wavelengths. BVR activity has been previously determined by incubating cell lysates with biliverdin and then measuring absorbance at 468 nm to determine the amount of bilirubin in the sample [13]. However, the absorbance method may not be sensitive enough for detection of minimum levels of BVR activity or the measurement of BVR activity in small quantities of cells or tissues. Also, analysis of intracellular bilirubin levels has not been possible. Thus, there is a need for sensitive methods to determine BVR activity in which starting materials or activity may be sub-optimum. Recently, a bilirubin-specific fatty-acid binding protein (UnaG) which fluoresces explicitly when bound to bilirubin was described in the Japanese freshwater eel (*Anguilla japonica*) [14]. We have adopted traditional absorbance-based BVR assays to incorporate monitoring of BVR activity via bilirubin-dependent UnaG fluorescence. This new assay exhibits greater sensitivity and adaptability as compared to the conventional absorbance-based methods for the measurement of BVR activity.

2. MATERIALS AND INSTRUMENTS

2.1. Materials

(1) Freshly collected liver from control and liver-specific BVRA-KO mice [7]. The experimental procedures and protocols of all animal studies conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

(2) Mouse cortical tubule (MCT) cell line (control) [15], BVRA knockout (KO), and BVRA-KO transduced with empty QCXIP vector or mouse BVRA (QCXIP-vector and QCXIP-BVRA).
(3) Hepa1c1c7 mouse hepatocyte cell line; control and BVRA-KO.
(4) Purified UnaG: It was obtained by cloning UnaG (AB731138.1), a gift from Dr. Atsushi Miyawaki, into pET-32a(+) vector (#69015; EMD Millipore, MA, USA), expressed in NiCo21 (DE3) bacteria (#C2529H; NEB, MA, USA), and purified with Ni-NTA Fast Start kit (#30600; Qiagen, MD, USA).
(5) Biliverdin hydrochloride (B655-9; Frontiers Scientific, UT, USA).
(6) Bilirubin-alpha (B584-9, Frontiers Scientific).
(7) β-NADP (N0505; Sigma-Aldrich, MO, USA).
(8) Protease inhibitor (P8340; Sigma-Aldrich).
(9) Phosphatase inhibitor (P5726; Sigma-Aldrich).
(10) BVR assay buffer: 100 mM Tris base, 1 mM ethylenediaminetetraacetic acid in distilled water, adjust pH to 8.7 before making up with distilled water to final volume. Store at 4ºC.
(11) RIPA buffer: 100 ml of 10× phosphate-buffered saline (PBS), 5 g sodium deoxycholate, 1g sodium dodecyl sulfate (SDS), 10 ml IGEPAL CA-630 made up to 1 liter with distilled water.
(12) IPTG dioxane-free (V3951; Promega, WI, USA).

2.2. Major Instruments

(1) Biorad Benchmark microplate reader (Hercules, CA, USA)
(2) Biotek Synergy H4 plate reader (Winooski, VT, USA)
(3) Eppendorf 5414R centrifuge (Hauppauge, NY, USA)

3. PROTOCOL AND STEPS

3.1. Protein Lysate-Based BVRA Assay

3.1.1. Preparation of Reagents

(1) A 6 M stock solution of biliverdin was prepared (in methanol) and stored at –20°C.
(2) Stock bilirubin was prepared at 5 mg/ml and stored at –20°C.
(3) Bilirubin standards were made fresh for each assay as 2× concentrations in PBS (0, 20, 200, 2000, and 20,000 nM).
(4) β-NADP was made fresh for each assay at 16 mg/ml in BVR assay buffer.
(5) RIPA buffer supplemented with protease and phosphatase inhibitors (RIPA-pp). Both inhibitors were used at a concentration of 1:100.
(6) Putative BVR inhibitors closantel and ebselen (#ab143384 and #ab142424; Abcam, Cambridge, UK) were made in dimethyl sulfoxide (DMSO) as 10 mM stocks and stored at –20°C.
(7) UnaG protein: Glycerol stock of the UnaG plasmid (pET-32a(+)-UnaG) expressed in NiCo21 (DE3) was inoculated into 6 ml culture medium containing 100 µg/ml ampicillin, and was cultured overnight. This culture was then transferred into 500 ml of ampicillin-supplemented culture medium and incubated with shaking. The growth was monitored every 30 min until OD_{600} of 0.4–0.6 was reached. The culture was then induced with IPTG at a final concentration of 200 µM. After further incubation for 5 h, the culture was centrifuged at 5000 *g* for 10 min. The protein was then purified with the Ni-NTA Fast Start kit according to manufacturer instructions.

We have also successfully used crude protein lysate of the bacteria without the protein purification kit to detect bilirubin concentrations in samples and standards. This was done by lysing the bacteria pellet with RIPA-pp (10 ml for pellet from 500 ml culture). The lysis was done for 1 h at room temperature with intermittent shaking. The mixture was then centrifuged at 16,000 *g* in an ultracentrifuge and the supernatant collected and stored at 4°C. The required amount of the cleared supernatant for detecting bilirubin concentration in a 200 µl volume will need to be determined empirically since the exact amount of UnaG in the mix cannot be determined. 10 µl of crude lysate in 200 µl of sample/standard is a good starting point. The right amount should differentiate between 10 nM bilirubin and blank and should produce a linear increase in fluorescence up to at least 1000 nM.

3.1.2. Steps

(1) Harvest cells/tissues and prepare lysates: Remove growth medium and wash with cold PBS to remove the remaining traces of medium. Lyse

cells with RIPA-pp (depending on protein yield from cells, 1 ml is a good starting point for a confluent 10-cm plate). Prepare tissue lysates in RIPA-pp (use 10 µl/mg wet weight of tissue and homogenize). Centrifuge lysate/homogenate at 16,000 *g* to clear and determine protein concentration of the supernatant by standard procedures.

(2) Add the required amount of protein (300–400 µg) into 1.5 ml Eppendorf tubes and make up to 250 µl with BVR assay buffer. Make a (no-protein) blank reaction tube with just RIPA-pp and BVR assay buffer.

(3) Add 250 µl of bilirubin 2× standards (0, 20, 200, 2000, and 20,000 nM) into another set of 1.5 ml Eppendorf tubes.

(4) Prepare BVR assay (2×) master-mix as follows: biliverdin stock (6 M), use 1:100 (= 60 µM final concentration in 2× master-mix); β-NADP stock (16 mg/ml), use 1:10 (= 1.6 mg/ml in 2× master-mix); UnaG (70 µg/ml final concentration in 2× master-mix). Make up to the final volume with BVR assay buffer. For example, 5 ml of the master-mix, sufficient for 20 samples/reactions (250 µl of the 2× master-mix will be used per reaction) will contain 50 µl biliverdin stock, 500 µl β-NADP stock, and 350 µg of UnaG made up to 5 ml with BVR assay buffer. Remember to prepare a few extra to make up for volume loss through repeated pipetting.

(5) Prepare UnaG solution for standards: 70 µg/ml in BVR assay buffer. 1.5 ml is usually enough for this since there are five standards.

(6) [For assays involving inhibitors, DMSO, closantel or ebselen, 1–2 µl of appropriate stock concentrations were added on the walls of the tubes before the master mix was added]. Add 250µl of the 2× master-mix (prepared in Step 4) to each of the 250 µl protein lysate samples and the blank. Vortex and incubate in the dark at room temperature or at 37ºC.

(7) Start timing.

(8) Add 250 µl UnaG solution for standards (prepared in Step 5) to each tube of the standards. Vortex and incubate along with the samples.

(9) Vortex the tubes again and load 200 µl of each of the reactions, blank, and standards twice in a flat-bottom, 96-well plate (black plates with transparent flat bottom is recommended for fluorescence measurement because of reduced background and cross-talk among adjacent wells).

(10) Read plates in a fluorometer with the following settings: excitation 485/20 nm; emission 528/20 nm. Read every 30 min at least twice and then every h thereafter (depending on the desired resolution and whether bilirubin production keeps increasing). Up to 3–6 h should be sufficient (**Figure 1B–1D**). Export the data to Microsoft Excel (WA, USA).

3.1.3. Calculation

Plot the standard concentration (x-axis) against the fluorescence (y-axis) after zeroing the fluorescence of 0 nM by subtracting the fluorescence value of the 0 nM standard (0 nM_{FL}) from fluorescence values of all the standards. The plotted graph should then be made to start from origin (0,0) (**Figure 1A**). The slope of this standard curve will be used in the calculation below.

In most cases as required, we express the results in nmol of bilirubin produced per microgram of protein used (nmol/µg protein = µmol/mg protein). The formula is: [Total quantity of bilirubin produced (BR_{QTY})] ÷ [Total quantity of protein used ($Protein_{QTY}$)], where, BR_{QTY} (in nmol) = [500 × ($Sample_{FL}$ – $Blank_{FL}$)] ÷ [Slope of Standard curve]. Note that 500 µl is the total volume of the reaction.

And $Protein_{QTY}$ (in µg) = [Protein concentration (µg/µl)] × [Volume used (µl)].

The exact BVRA activity (in nmol/µg/min = µmol/mg/min) can be determined from the slope. As shown in **Figure 1B–1D**, BVR activities were clearly higher in control mouse liver (**1B**), MCT cells (**1C**), and Hepa1c1c7 cells (**1D**) compared to their respective BVRA-KOs.

3.1.4. Other Considerations

It is important to note that standards must be prepared for each assay. Standards and blank should be read at every time point to determine the amount of bilirubin produced by each sample at the corresponding time points. Also note that the final standard concentrations are 0, 10, 100, 1000, and 10,000 nM.

3.2. In-Cell BVR Assay

This assay format is based on an approach to assess the BVR activity in a physiological condition. This is most applicable to cell culture experiments. We ini-

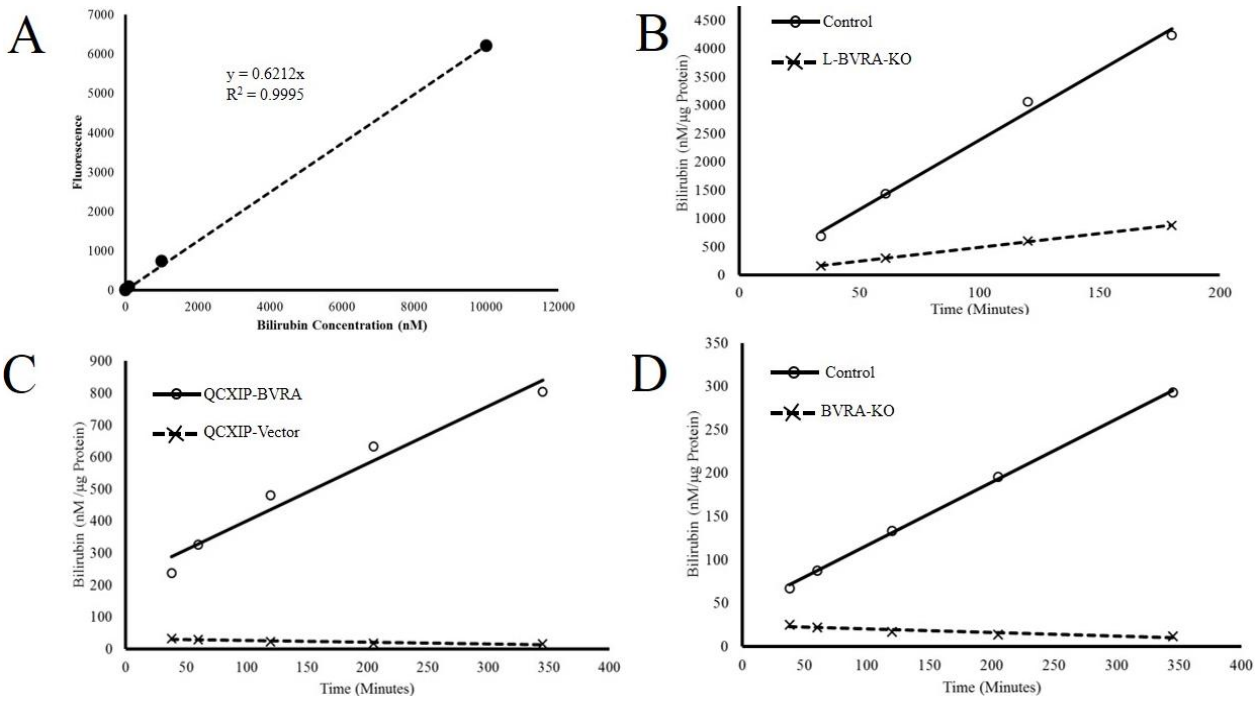

FIGURE 1. Bilirubin standard curve with UnaG and lysate-based BVR assay. (A) Standard curve with various concentrations of bilirubin in PBS detected with 35µg/ml (final concentration) of purified UnaG. (B) BVR assay with 400 µg of lysates of liver tissues from control (y = 24.5x – 69.0) and liver-specific BVRA-KO (L-BVRA-KO) mice (y = 4.9x – 0.8). Lysate-based BVRA assay with 400 µg of (C) BVRA-KO (QCXIP-vector; y = -0.04x – 24.9) and BVRA-overexpressing (QCXIP-BVRA; y = 1.4x – 179.5) MCT cells and (D) Control (y = 0.6x – 34.4) and BVR-KO (y = -0.03x – 19.2) Hepa1c1c7 cells. For B-D, 3 independent reactions (read in duplicates) were set up for each of the cell lines. The assays were set up as described in Protocol and Steps (Section 3.1). The bilirubin production was monitored with UnaG included in the reaction.

tially used an overnight incubation, but found that this duration (16–24 h) was too long and not very accurate for an endpoint assay format. This is primarily because of substrate (biliverdin) depletion. While it is possible to simply increase the amount of biliverdin available for conversion, toxicity from long term exposure to bilirubin is a concern. Moreover, our aim was to establish a simple, sensitive, and fast assay. Thus, we first optimized the time and concentration that can be used (**Figure 2**). From the data obtained, we determined that 1 h exposure to 10 µM biliverdin is optimal. Hence these conditions were used to determine BVR activity in all MCT cell types which have normal, high, or no BVRA expression (**Figure 3A–3C**) and in control and BVRA-KO Hepa1c1c7 cells (**Figure 3D**).

3.2.1. Preparation of Reagents

(1) 10 mM biliverdin stock was prepared in DMSO (6.19 mg/ml) and stored at –20°C.

3.2.2. Steps

(1) Thaw the 10 mM biliverdin stock and make a 1:1000 dilution (10 µM) in pre-warmed media.
(2) [For assays involving inhibitors, pre-treated cells with DMSO, closantel, or ebselen for 16–24 h]. Aspirate the spent medium on the cells and replace with the right volume of 10 µM biliverdin medium.
(3) Incubate at normal growth condition (5% CO_2 at 37°C) for 1 h.

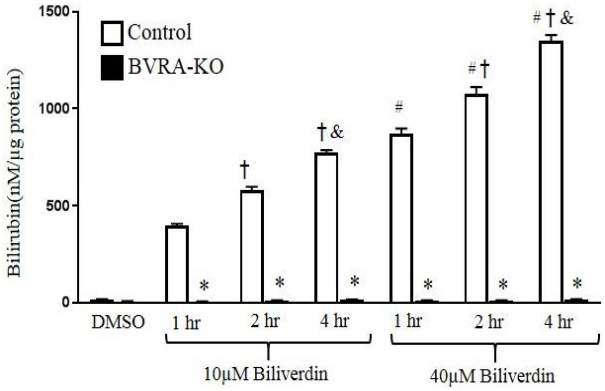

FIGURE 2. Time and concentration optimization for in-cell BVR assay. Control and BVRA-KO MCT cells were treated with 10 µM or 40 µM biliverdin for 1, 2, or 4 h. After washing with PBS, cells were lysed with RIPA buffer and bilirubin content (expressed in nmol/µg protein) were determined as described in Protocol and Steps (Section 3.2.2). Bars represent mean ± SEM. *, $p < 0.05$ as compared to control. †, $p < 0.05$ as compared to 1 h treatment. &, $p < 0.05$ as compared to 2-h treatment. #, $p < 0.05$ as compared to corresponding value in 10 µM treated. n = 3/group.

(4) Aspirate the biliverdin medium and wash wells with excess cold PBS.
(5) Lyse cells within the well with cold RIPA buffer (130 µl/well for 24-well plates; scale up or down as required) and incubate on ice for 10 min.
(6) Clear the supernatant by centrifugation.
(7) Prepare bilirubin standards and UnaG solution (70 µg/ml). Note that the same UnaG solution will be used for all the samples, standards, and blank.
(8) Transfer 100 µl of the standards, lysate, and RIPA buffer (blank) into wells of 96-well plates.
(9) Determine protein concentration of each sample with the remaining lysate.
(10) Add 100 µl of UnaG solution into each well. Use a multichannel pipette if working with many samples.
(11) Incubate at room-temperature or 37°C for 30–60 min.
(12) Read once in the fluorometer with the setting as in Step 10 in Section 3.1.2.

3.2.3. Calculation

Calculation is as in Section 3.1.3 above. However, note that since the concentration of bilirubin was determined in 200 µl reaction volume and 100 µl of cell lysate is used:

BR_{QTY} (in nmol) = 200 × [($Sample_{FL}$ – $Blank_{FL}$) ÷ Slope of Standard curve].

$Protein_{QTY}$ (in µg) = Protein concentration (µg/µl) × 100 (µl).

3.2.4. Other Considerations

It may be necessary to determine the optimal concentration and time for different cell lines, depending on the expression of BVRA. For example, as low as 0.4 µM biliverdin for 1 h differentiated between the BVRA-KO (QCXIP-vector) cells and QCXIP-BVRA cells which expresses a much higher level of BVRA than control cells (**Figure 3C** and **3A**).

3.3. Determination of Intracellular Bilirubin Content (iBR)

3.3.1. Preparation of Reagents

None needed.

3.3.2. Steps

(1) On the day before the assay, change medium on confluent cells to serum-free medium.
(2) On the day of the assay, remove medium and wash with excess PBS.
(3) Scrape cells into cold PBS and collect cells by centrifuging at 500 *g* for 5 min.
(4) Remove the PBS.
(5) Lyse cells with RIPA buffer (230 µl per 6-cm dish).
(6) Continue as in Step 6 in Section 3.2.2.

3.3.3. Calculation

The calculation is the same as that described in Section 3.2.3 above.

3.3.4. Other Considerations

One of the major considerations is that a larger quantity of cells will be needed. Another major considera-

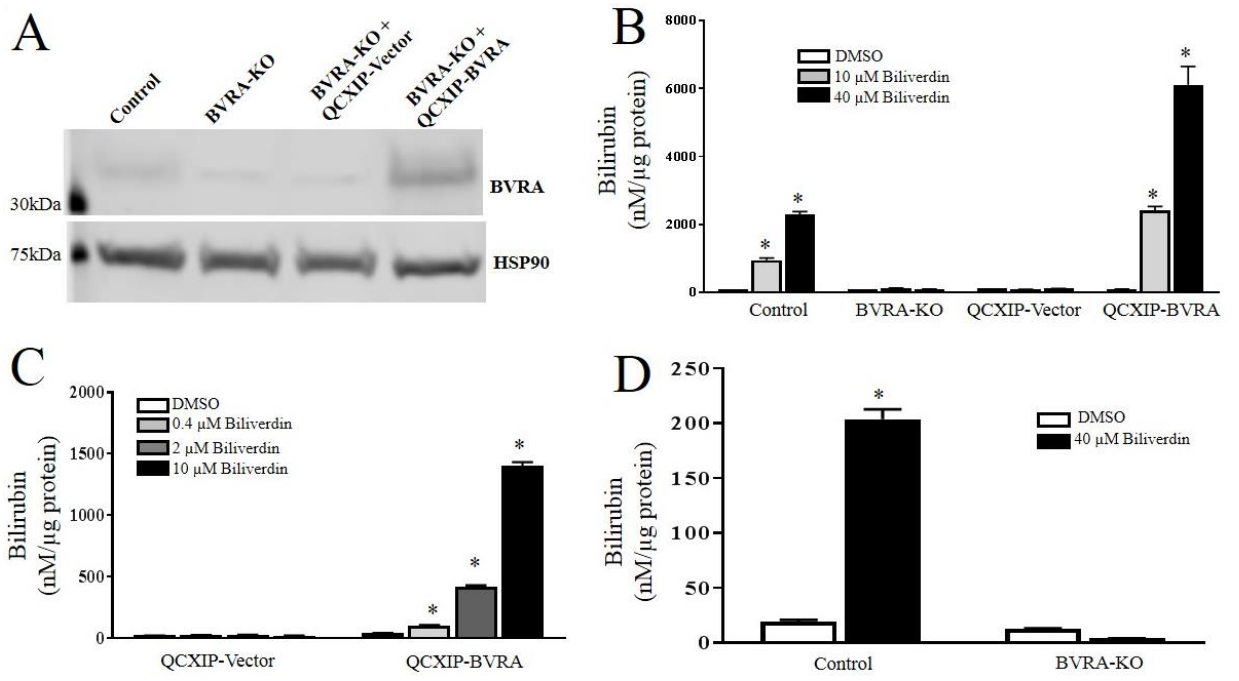

FIGURE 3. In-cell BVR assay in MCT and Hepa1c1c7 cells. (A) Western blot showing BVRA expression in the different MCT cells used. (B) In-cell BVR assay of the different MCT cells using 10 µM or 40 µM biliverdin for 1 h. (C) In-cell BVR assay in BVRA-KO (QCXIP-vector) and BVRA-overexpressing (QCXIP-BVRA) MCT cells with lower concentrations of biliverdin and 1 h incubation. (D) In-cell BVR assay in control and BVRA-KO Hepa1c1c7 cells. For B-D, following treatment, cells were washed with PBS, lysed with RIPA buffer and bilirubin content (expressed in nmol/µg protein) were determined as described in Protocols and Steps (Section 3.2.2). Bars represent mean ± SEM. *, $p < 0.05$ as compared to DMSO treated. n = 3/group.

tion is the significant amount of bilirubin found in commonly used types of cell culture serum supplements (**Figure 4A**). As such, it will be necessary to incubate the cells with serum-free medium for several h to overnight, in order to remove this confound. Using this method, we were able to measure bilirubin content of MCT cells cultured in 6-cm dishes (**Figure 4B**). It is also worth noting that the cells should be harvested by scrapping into PBS and not with trypsin which will require a medium with the serum to neutralize trypsin.

4. DISCUSSION OF ADVANTAGES AND LIMITATIONS

There is a need for more fine-tuned assays measuring bilirubin levels, as well as determining its intracellular concentrations. Traditional BVR assays rely on the measurement of bilirubin absorbance with the lower sensitivity in the range of 100–500 nM bilirubin. Our novel UnaG based assay can detect bilirubin as low as 1 nM. The high sensitivity of our newly described UnaG-based assay is required for the limited amount of starting materials that researchers have to work within typical cell culture experiments. The enhanced sensitivity of our UnaG-based test is ideal for high throughput since analyses can be done in 24-well plates rather than 10-cm cell culture dishes which are necessary to obtain sufficient quantities of lysates for traditional absorbance-based BVR assays. The primary factor responsible for the increased sensitivity is the use of the fluorescent protein, UnaG, which we have incorporated into the conventional absorbance-based BVR assay [13]. This method also negates the need to extract biliru-

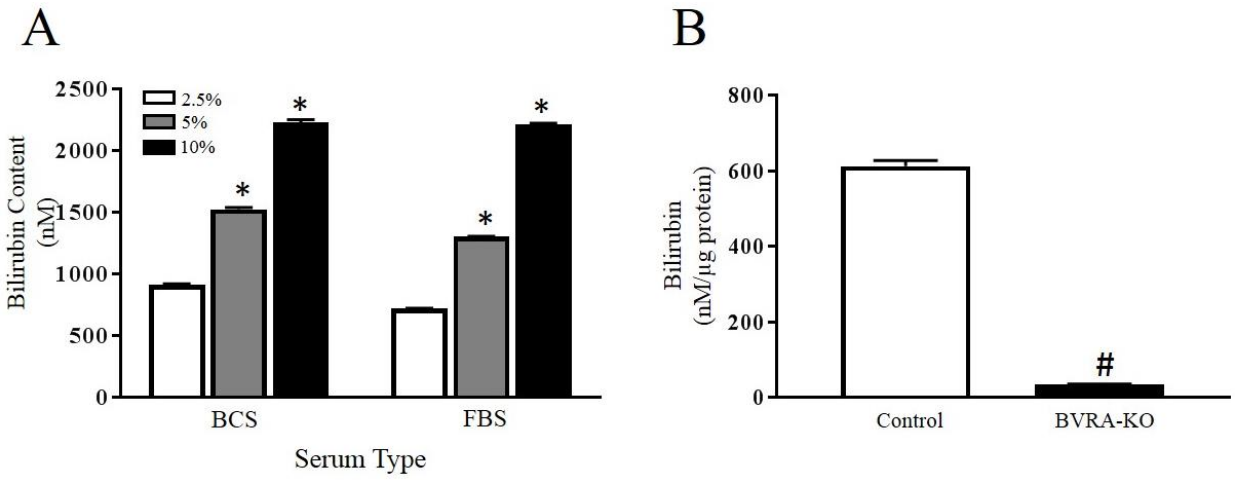

FIGURE 4. Bilirubin content in typical cell culture serum and intracellular bilirubin content in MCT cells. (A) Bilirubin content of bovine calf serum (BCS) and fetal bovine serum (FBS) determined in various dilutions of the sera in PBS. (B) Bilirubin content of control and BVRA-KO MCT cells grown overnight in 6-cm plates with serum-free (DMEM-F12) medium. Bars represent mean ± SEM. *, $p < 0.05$ as compared to 2.5%. #, $p < 0.05$ as compared to control. n = 3/group.

bin before measuring absorbance which is required in traditional absorbance-based BVR assays [13]. Our UnaG-based BVR assay allows for repeated measurements of bilirubin production in the same reaction sample over time without stopping the reaction. One advantage is that data from repeated measures will allow for the monitoring of bilirubin produced over time to generate BVR activity curves as compared to the traditional absorbance-based assay which stops the reaction and bilirubin production is only measured at a single time point.

The novel in-cell BVR assay described in Section 3.2 has advantages in assessing BVR activity at physiological conditions or after administration of BVR inhibitors or activators which is impossible with the traditional lysate/absorbance-based assay [13]. Another advantage of the in-cell based UnaG BVR assay is that the analysis can be completed within 1 h. This aspect of our novel UnaG-based test can be adapted for high throughput fashion screening of novel BVR inhibitors or inducers. Cells could be treated with BVR inhibitors or inducers and the reductase activity measured by treating the cells with biliverdin during the last hour (see Step 2 in Section 3.2.2) of the drug treatment and then monitoring UnaG based fluorescence. This approach would reduce the likelihood of false positives (molecules that interfered with the chemical reaction/milieu in the lysate-based assay) and false negatives (molecules that need prolonged exposure to inhibit or induce BVR).

We tested two of the putative BVRA inhibitors identified in a recent drug screen [16]. The lysate-based assay employed in this study showed that both closantel and ebselen (10 µM) inhibited BVRA activity by more than 90% [16]. We tested both inhibitors in our in-cell assay and found that while closantel inhibited BVRA significantly at 5 µM (higher concentration was toxic to cells), ebselen did not, even at 40 µM (**Figure 5A**). Interestingly, ebselen but not closantel inhibited BVRA in the lysate-based assay (**Figure 5B**). This difference in the effects of these inhibitors in the two different assays highlights the importance of testing potential BVR inhibitors in the most physiologically relevant conditions. It is possible that the ebselen works in cell lysates through a change in the chemical milieu of the assay limiting the formation of bilirubin rather than inhibition of BVR per se. It is also possible that ebselen is not able to act on BVR in intact cells through metabolism or an inability to access BVR. Closantel was effective in inhibiting BVR activity using the in-

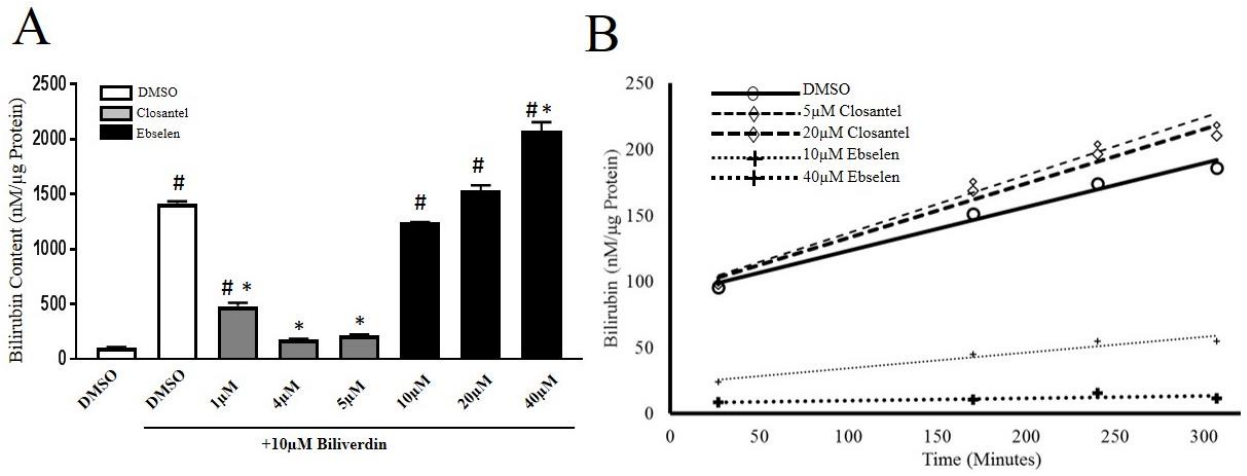

FIGURE 5. Effects of BVR inhibitors in lysate-based and in-cell BVR assays. (A) In-cell BVR assay as described in Section 3.2.2 after 23 h pretreatment of control MCT cells with DMSO or various concentrations of putative BVR inhibitors (closantel and ebselen). Bars represent mean ± SEM. n = 3 in all bars. (B) Lysate-based BVR assay as described in Section 3.1.2 in the presence of various concentrations of putative BVR inhibitors (closantel and ebselen). The slopes are measures of BVR activity. (DMSO: y = 0.33x + 90.3; 5 µM closantel: y = 0.44x + 92.8; 20 µM closantel: y = 0.41x + 91.8; 10 µM ebselen: y = 0.12x + 22.8; 40 µM ebselen: y= 0.02x + 8.3). #, $p < 0.05$ as compared to DMSO alone. *, $p < 0.05$ as compared to DMSO + biliverdin. n = 3/group.

cell assay but not when used with cell lysates. This suggests that closantel may either be metabolized to an active form in intact cells or inhibits the production of bilirubin through a mechanism which is lost upon disruption of the cellular environment.

The main limitation of these assays is interference from potentially fluorescent contents of the samples which could alter the UnaG-based fluorescent signal. For example, when tissues are not well perfused, blood can interfere with the UnaG-based fluorescent signal. This can be circumvented by adequate perfusion of tissues with PBS-based solutions prior to collection or further dilution of tissue samples when measuring the BVR activity. However, when measuring bilirubin content directly from tissues, further dilution of samples may not be helpful as it may reduce the bilirubin content below the detection limit of the UnaG-based assay (< 1 nM). Another limitation is that these methods cannot be used for tissues or cells expressing green fluorescent protein (GFP) or related fluorescent proteins that also interfere with UnaG-based fluorescence in the needed excitation/emission wavelengths.

5. CONCLUSION

BVR is the enzyme responsible for the last step in the production of bilirubin which is one of the most potent antioxidant molecules in the body [3, 17]. Monitoring BVR activity is necessary to determine the antioxidant capacity of cells and tissues. We have modified and improved the traditional method of measuring BVR activity based upon the determination of bilirubin production by absorbance at 468 nm to incorporate measurement using the bilirubin-inducible fluorescent fatty-acid binding protein UnaG [14]. Incorporation of the fluorescent UnaG protein significantly increased the sensitivity of the BVR assay as well as allowed for serial measurements of bilirubin production from single tests as well as for the determination of BVR activity intracellularly and levels in cells and tissues. The fluorescence-based analysis can also be adapted for the high throughput screening of BVR activity in response to treatment with inhibitors and activators. The UnaG method of detection of bilirubin is a significant improvement over the traditional absorbance-based

BVR assay and will help in the measurement of BVR activity and intracellular bilirubin levels in a wide range of experimental settings.

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