

Identification of Bakuchiol Targeting Proteins in Human Skin Cells

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Abstract: Purpose: Bakuchiol is a natural product that is widely used for skin antiaging. Previous studies show that bakuchiol and retinol have similar biological activities despite having no structural resemblance, but the mechanism of action of bakuchiol remains largely unknown. We proposed a regulatory pathway of bakuchiol that is based on the downstream activities of retinol. In this work, we will test a small portion of the proposed pathway—that is, bakuchiol can bind to target proteins different than that of retinol (RARs). Methods: We will design experiments to identify the molecular targets of bakuchiol. We will perform affinity chromatography to isolate bakuchiol targeting proteins, followed by mass spectroscopy to sequence the target proteins. The sequences will be identified using a database. Possible Results: At the end, there are three possible results: (1) Bakuchiol does not bind the target proteins of retinol (RARs) and instead binds other proteins; (2) Bakuchiol only binds RARs. (3) Bakuchiol binds RARs and other proteins. Conclusion: The results of our study will pave the way for understanding the mechanism of action of bakuchiol.

1 INTRODUCTION

Bakuchiol is a natural product mainly obtained from the seeds of the plant *Psoralea corylifolia*. Previous studies have shown that bakuchiol can serve as a functional analog to retinol even though bakuchiol does not look like a retinoid (Figure 1). For example, retinol has long been used as a therapeutic agent to treat photo-aged human skin. Similarly, Chaudhuri and Bojanowski showed through a pilot clinical study that the clinical appearance of photo-aged human skin is improved by bakuchiol, similar to retinol (Chaudhuri, Bojanowski 2014). The functional similarity of bakuchiol and retinol is also reflected in, for example, their antioxidant and antiinflammation effects (Chaudhuri, Sivamani, Jagdeo, Elsner, Maibach 2015). To investigate the similarity of bakuchiol and retinol on a molecular biology level, the authors applied a comparative gene expression profiling with both bakuchiol and retinol by using the technique of DNA microarray (Chaudhuri, Bojanowski 2014). They revealed that the volcano plots of the two substances are similar in shape (Figure 2), indicating their similar regulation on gene expression. This leaves a question on why bakuchiol and retinol, having no structural resemblance with each other, regulates gene expression in a similar pattern. One possibility is that bakuchiol binds to the

same targets as that of retinol (i.e., retinoic acid receptors, or RARs). Another possibility is that bakuchiol binds to different targets than retinol, which somehow improves the availability of endogenous retinol. The latter possibility is supported by Chaudhuri and Bojanowski's observation that bakuchiol upregulates proteins (CRBP II, CRBP IV, CRABP I, LRAT) that can help increase the cellular storage of retinol to an extent greatly higher than that of retinol (Chaudhuri, Bojanowski 2014). This indicates a possible regulation pathway that bakuchiol achieves its functional similarity to retinol by binding to protein targets different than that of retinol; the targets directly or indirectly activate transcription factors that mediate the expression of the retinol-storage-related proteins, which increases the availability of endogenous retinol. In other words, bakuchiol increases available retinol in cells, and it is the increased retinol that ultimately acts to exhibit the anti-aging activity of bakuchiol in human skin cells.

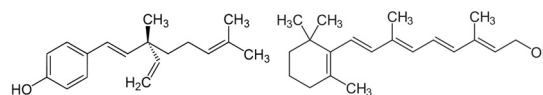


Figure 1: Structure of bakuchiol (left) and retinol (right).

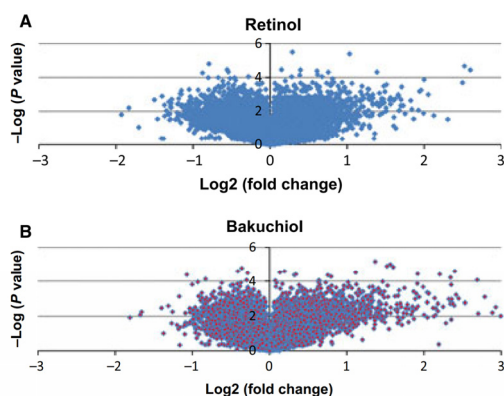


Figure 2. Volcano plots of DNA microarray data obtained from retinol- (A) and bakuchiol- (B) treated skin substitutes. The overall shapes of the two volcano plots are similar, indicating that retinol and bakuchiol have similar regulation on gene expression.

However, more evidence is still needed to support this hypothesized pathway. It is also not clear what are the targets of bakuchiol and how exactly they regulate the expression of the retinol-storage-related proteins. In this study, I will test a small portion of the hypothesized pathway—bakuchiol can bind to different targets than retinol. To test this hypothesis, I will design an experiment to identify the bakuchiol targeting proteins (BTPs) in cells.

I will perform bakuchiol affinity chromatography on human dermal fibroblast lysates, followed by mass spectrometry to sequence the BTPs. The use of affinity chromatography assumes that modifications at the 4-hydroxyl group of bakuchiol do not significantly alter its anti-aging biological activity in human skin cells. To test the assumption, I will perform a structure-function study by substituting the 4-hydroxyl group of bakuchiol with methoxy group and then comparing the biological activity of bakuchiol and 4-methoxy-bakuchiol in stimulating the expression of collagens by ELISA.

The result will provide hints for future researchers to investigate the mechanism of action of bakuchiol.

2 MATERIALS AND METHODS

2.1 Structure-Function Study

2.1.1 Materials

Bakuchiol (INCI name), also known as Sytenol A (trade name), will be purchased from Sytheon. Sytheon derives bakuchiol from the plant *Psoralea*

corylifolia, which contains edible seeds that serve as the source of bakuchiol. The plant itself is psoralene-depleted bakuchiol with a purity of about 95%. The Williamson-Ether method will be used to generate methoxy-bakuchiol.

2.1.2 Synthesis of Methoxy-Bakuchiol

The Williamson-Ether method used to generate methoxy-bakuchiol is as follows: 0.9 mmol/L bakuchiol in acetone, treated with 4.8 mmol/L methyl iodide, and 3.6 mmol/L potassium carbonate. The solution will be stirred and refluxed for 24 hr; afterward, there will be further room temperature stirring for an additional 72 hr. The reaction will be followed with thin-layer chromatography (25 ml of methyl chloride and 3 ml of methanol). The acetone and excess methyl iodide will be removed with a stream of N₂ gas. The remaining solid will be resuspended in approximately 50 ml of ether and vacuum filtered to remove the potassium carbonate. The ether will be removed with N₂ gas, leaving an oily, yellow residue behind. This residue will be resuspended in 50 ml of methanol and triturated with water, then refrigerated (4°C) overnight to produce pale beige crystals of methoxy-bakuchiol. The structural confirmation of methoxy-bakuchiol will be determined through infrared spectrum (Nicolet IR spectrometer, in KBr pellet) and a proton nuclear magnetic resonance spectrum (Varian UnitccccPlus, 400 MHz, in DMSO). A sample of methoxy-bakuchiol will be submitted for elemental analysis (Desert Analytical, Tucson, AZ). The purity of the sample will be assessed in two ways: 1) by measuring its melting point, R_f on thin layer chromatography; 2) by using high performance liquid chromatography (Beckman Gold) that incorporates an isocratic 0.1% trifluoro acetic acid:acetonitrile (50/50) solvent system and a C18 reverse phase column at 0.8 ml/min.

2.1.3 Collagen ELISA

Bakuchiol and methoxy-bakuchiol will be assayed at 10 µg/mL on normal human fibroblasts grown in DMEM with 5% calf serum (Hyclone, Salt Lake City, UT, U.S.A.). Neonatal human dermal fibroblasts will be used in analysis of Type I and IV collagen quantification (low passage; American Type Culture Collection, Manassas, VA, U.S.A. cat. no. PCS-201-010, lot no. 58243223). Human epidermal fibroblasts from a 68-year-old female donor will be used in analysis of Type III collagen quantification (p. 5, Zen-bio, cat. no. KR-F). Type I collagen quantification requires cells to be subjected to test

materials for 3 days, whereas Type III and IV collagen quantification requires cells to be subjected to test materials for 7 days. Afterward, per standard ELISA protocol, the sandwich ELISA, which uses affinity-purified antibodies, will be used to assay the collected cell culture conditioned media for type I, type III or type IV collagen, which is then followed by streptavidin-avidin-HRP conjugate and ABTS (Dobak, Grzybowski, Liu et al 1994, Zhao, Alexeev, Chang, et al. 2005). The collagen content is proportional to a colorimetric signal. A BioRad microplate spectrophotometer 3550-UV at 405 nm with background subtraction at 660 nm will be used to measure this colorimetric signal. Further analysis of this colorimetric signal will be performed with Microplate Manager v.2 software for Macintosh (BioRad, Hercules, CA, U.S.A.).

2.2 Affinity Chromatography and Mass Spectroscopy

2.2.1 Materials

Epoxy-activated agarose resin (12 atom linker, 33 μmol of epoxy group/ml of packed gel) will be purchased from Sigma Chemical (St. Louis, MO). Bakuchiol stock at a concentration of 12.5 mM will be made in DMSO and stored at -20°C . Other biochemical reagents will be procured from a variety of chemical suppliers. The National Cell Culture Center in Minneapolis, MN will source the large amounts of cultured fibroblasts.

2.2.2 Preparation of Immobilized Bakuchiol Affinity Column (BAC)

1 g of epoxy-activated agarose will be held in ice-cold water for 5 min and thoroughly washed to get rid of any additives or impurities. 23 mg of Bakuchiol dissolved in 2.5 ml of 0.1 M NaOH will be added and incubated with 1 ml of resuspended epoxy-activated agarose for a night at room temperature to ensure that the resin binds to the bakuchiol. 6 ml of 1 M sodium acetate buffer (pH 5.0) containing 1 mM dithiothreitol (DTT) will be added to the mixture to neutralize unreacted epoxy groups and eliminate further bakuchiol oxidation, thus halting the reaction. Immobilized bakuchiol resin will be washed successively with 0.1 M sodium acetate, pH 5.0, containing 1 mM DTT and 70%, 30%, 10%, and 0% ethanol, respectively. Mock-treated beads (which utilize identical procedure except that there is no added bakuchiol) or beads immobilized with a tyrosine ligand will make up the controls.

2.2.3 Fractionation of Cytoplasmic Extracts on BAC

Cultured mammalian cells will be lysed by 3 freeze-thaw cycles with buffer containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM Mg (OAc)₂, 1 mM DTT, 0.5% NP-40, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 $\mu\text{l/ml}$ of the protease inhibitor cocktail sourced from Sigma Chemical. 10-min centrifugation using a refrigerated microcentrifuge will yield the cell-free extracts.

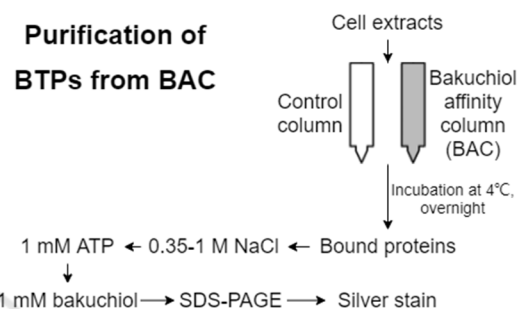


Figure 3. Purification strategy for the isolation of BTPs from BAC. In step 1, a cell lysate is passed over BAC or a mock-coupled or tyrosine-linked control column in parallel, and washed exhaustively with lysis buffer to remove nonspecific proteins. In step 2, the column is eluted stepwise eluted with 0.35 and 1 M NaCl. In step 3, column-bound proteins are eluted using 1 mM ATP. The last step involves elution with 1–2 mM bakuchiol dissolved in 2% DMSO.

To characterize BAC (Figure 3), 200 μl fibroblast extract containing 0.6–1.0 mg protein will be combined with 50 μl control (mock-treated or tyrosine-linked) or bakuchiol-immobilized agarose beads in a 1.5-ml Eppendorf tube. The tube will be stored overnight at 4 $^{\circ}\text{C}$ using a modest tumbling process. The protein extract, which resembles a gel slurry, will be loaded onto a minicolumn (from Pierce Chemical) and rinsed with 10–20 ml lysis buffer to eliminate any proteins that did not successfully bind. Elution of the column will be next occur 5–7 times, each time with 0.5 ml lysis buffer containing 0.35 M NaCl, and will be followed by the same number of rinsing using 1 M NaCl supplemented buffer. Next, the column will be equilibrated with the lysis buffer and eluted with 1 mM ATP. For the final step, the column will once more be re-equilibrated with the lysis buffer and eluted with 1–2 mM bakuchiol dissolved in 2% DMSO.

2.2.4 Using MALDI-TOF MS to Identify BTPs from Fibroblasts

We will use fibroblasts to prepare cytoplasmic extracts which will be fractionated as described (Figure 3). The middle silver-stained protein bands will be excised, reduced, carbamidomethylated, and cleaved with trypsin. The subsequent peptide mixture will be desalted and concentrated using Zip-Tip C18 micro-columns (Millipore), and applied to the MALDI target using solution phase nitrocellulose method, as defined by Landry et al. (Landry, Lombardo, Smith 2000). We will use a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) set in reflector mode with standard settings to calculate peptide masses. Trypsin autodigestion products will be used for internal mass calibration. MALDI-TOF MS generates peptide mass fingerprinting which will be compared to the theoretical tryptic digests of proteins stored in NCBI nonredundant protein database using the ProFound software (<http://prowl.rockefeller.edu/cgi-bin/ProFound>). This technique is used to identify proteins. The identity of the BTPs will be obtained from the database search result.

2.2.5 Statistical Analysis

The statistical significance of all numerical data will be analyzed using the student's T-Test on GraphPad Prism® at (p <0.05).

3 POSSIBLE RESULTS

3.1 Confirmation of the Applicability of Affinity Chromatography

Table 1 lists all the possible stimulatory effects of bakuchiol and 4-methoxy bakuchiol on collagen I, II, and IV.

Table 1. Possible stimulatory effects of bakuchiol and 4-methoxy bakuchiol on collagen I, II, and IV. Bakuchiol and 4-methoxy bakuchiol may have the same or different stimulatory effects on each of collagen I, II, and IV. The same stimulatory effect is defined as the two substances decrease or increase the concentration of a collagen by the same amount (p<0.05). In this table, the same stimulatory effect is represented by “+”, and different stimulatory effect is represented by “-”.

	Collagen I	Collagen III	Collagen IV
Possible result 1	+	+	+
Possible result 2	+	+	-
Possible result 3	+	-	+
Possible result 4	+	-	-
Possible result 5	-	+	+
Possible result 6	-	+	-
Possible result 7	-	-	+
Possible result 8	-	-	-

3.2 Identification of Molecular Target by Affinity Chromatography

Possible result 1: There is no silver-stained protein shown in the gel.

Possible result 2: There is at least one silver-stained proteins shown in the gel.

3.3 Identification of Molecular Target by Mass Spectroscopy

Possible result 1: Bakuchiol does not bind RARs.

Possible result 2: Bakuchiol only binds RARs.

Possible result 3: Bakuchiol binds RARs and other proteins.

4 DISCUSSION

4.1 Confirmation of the Applicability of Affinity Chromatography

To secure and detect BTPs, we will immobilize bakuchiol onto epoxy-activated agarose beads. Residual bakuchiol found on column will be eliminated with thorough rinsing. Chemical coupling of bakuchiol occurs at the 4-hydroxyl group, which yields a bakuchiol-immobilized affinity column (BAC). However, it is not guaranteed that the coupling between bakuchiol and epoxy-activated agarose beads does not significantly affect the antiaging biological activity of bakuchiol. We therefore will perform a structure-function study to examine if modifications at the 4-hydroxyl group of

bakuchiol alters the antiaging biological activity of bakuchiol.

We synthesized 4-methoxy bakuchiol and will compare its stimulatory effect with bakuchiol on the expression of collagens in skin model.

The main components of the skin extracellular matrix (type I and type III collagens) and basement membrane (type IV collagen) include collagens produced by dermal fibroblasts. Dermal fibroblasts are reduced in amount and quality as found in aged and photodamaged skin in addition to having less new collagen pool. Consequently, we choose to measure select collagens by ELISA method to confirm the assumption that modifications at the 4-hydroxyl group of bakuchiol does not alter the antiaging biological activity of bakuchiol.

It is known from Chaudhuri and Bojanowski's work that bakuchiol can stimulate the amount of collagen I, III, and IV in human dermal fibroblasts in vitro (Chaudhuri, Bojanowski 2014). We therefore choose collagen I, III, and IV to test our assumption. Possible results are summarized in Table 1.

Possible result 1 the most strictly indicates that modifications on the 4-hydroxyl group of bakuchiol does not significantly alter its biological effect. As a result, bakuchiol and 4-methoxy bakuchiol has the same stimulatory effect.

Possible results 2, 3, and 5 indicate that modifications on the 4-hydroxyl group of bakuchiol can significantly alter its biological effect. As a result, in the case of a substitution of 4-hydroxyl group with 4-methoxy group, the stimulation of one of the three collagens has changed compared to bakuchiol.

Possible results 4, 6, and 7 indicate that modifications on the 4-hydroxyl group of bakuchiol can alter its biological effect to a greater extent than that of possible results 2, 3, and 5. As a result, in the case of a substitution of 4-hydroxyl group with 4-methoxy group, the stimulation of two of the three collagens has changed compared to bakuchiol.

Possible result 8 indicates that the 4-hydroxyl group of bakuchiol is necessary for it to exhibit its biological effect, and that modifications on the 4-hydroxyl group of bakuchiol can the most greatly alter its biological effect. As a result, in the case of a substitution of 4-hydroxyl group with 4-methoxy group, the stimulation of all of the three collagens has changed compared to bakuchiol.

The alteration of the biological effect of bakuchiol as a result of modifications on the 4-hydroxyl group is most likely because the bakuchiol derivatives have different binding affinities with biomolecules in the cells. This can result in an alteration of the molecular

regulation, leading to an alteration of biological effects.

If possible results 2-8 occur, the subsequent affinity chromatography will not be applicable, because the immobilization of bakuchiol at the 4-hydroxyl group will affect the binding of bakuchiol to putative BTPs. Other experimental method should be designed to identify the molecular target of bakuchiol.

If possible result 1 occurs, we are confident that the subsequent affinity chromatography will be applicable. The reason is that, as possible result 1 indicates, the immobilization of bakuchiol at the 4-hydroxyl group will not affect the binding of bakuchiol to putative BTPs, and that the only position of bakuchiol for epoxy-activated agarose beads to bind is the 4-hydroxyl group. We can therefore claim that column-bound bakuchiol should maintain similar binding characteristics as bakuchiol in solution, and thus affinity chromatography should be an applicable method for the identification of the molecular target of bakuchiol in photo-aged human skin cells.

4.2 Identification of Molecular Target by Affinity Chromatography

Fibroblast lysates will be incubated with BAC and eluted, as indicated in Materials and methods. We will use SDS-PAGE to analyze eluted samples. Furthermore, we will use silver staining to visualize eluted samples. There may or may not be proteins that show specific retention on BAC.

Possible result 1: There is no silver-stained proteins.

The absence of silver-stained proteins suggests that bakuchiol does not bind to any molecular target in Fibroblasts. This is a very unlikely result given that it have been proved that bakuchiol does have biological affinity in photoaged human skin cells. If this result occurs, there might be human errors during the experiment, and further action should be done to examine, e.g., if the cell lysates have active proteins.

Possible result 2: There is at least one silver-stained proteins.

The presence of at least one silver-stained protein suggests that bakuchiol has molecular target(s) in fibroblasts, which bind to BAC and retain in the gel. It is likely that some of the targets are involved in the mechanism of action of the anti-aging activity of bakuchiol. Next, we will perform mass spectroscopy to reveal the identity of them.

4.3 Identification of Molecular Target by Mass Spectroscopy

Silver-stained proteins will be excised from the gel and subjected to in-gel trypsin digestion to generate peptide fragments that will be further determined by MALDITOF MS. This method will produce recognizable patterns that will be used in database searches to match predicted tryptic peptide masses of proteins with known identity, thus leading to determining what the silver-stained proteins are.

We hypothesized that bakuchiol binds to different molecular targets than that of retinol (RARs), but there are other possibilities.

Possible result 1: Bakuchiol does not bind RARs and instead binds other proteins.

This result is consistent with our hypothesis that bakuchiol can bind to different target proteins other than that of retinol. By binding to different target proteins, it is plausible that bakuchiol exhibits its biological activity by facilitating the intracellular storage of retinol, and it is retinol that ultimately function to achieve the biological activity of bakuchiol. This explains why bakuchiol has similar but not exactly the same gene regulation pattern as retinol, and why bakuchiol has antiaging activity as retinol does.

The hypothesized pathway proposed in the Introduction part of this paper suggests that there are transcription factors that mediate the enhanced retinol storage. Further studies should examine whether or not the identified targets are the above-mentioned transcription factors, or the identified targets can activate those transcription factors. It also needs to be clarified whether or not the above-mentioned transcription factors mediate the expression of retinol-storage-related proteins (e.g. CRBP II, CRBP IV, CRABP I, LRAT).

In addition, the hypothesized pathway may not be sufficient to describe the biological activity of bakuchiol. Maybe some bakuchiol targeting proteins facilitates cellular uptake and/or retinol activation and/or intracellular transportation of retinol. These possibilities also need to be tested in future studies.

Possible result 2: Bakuchiol only binds RARs.

This result is contradictive with our hypothesis. If bakuchiol only binds RARs, theoretically bakuchiol should have the same biological activity as retinol, which contradicts with the different gene expression pattern (Figure 2). Therefore, this is not a likely result.

Possible result 3: Bakuchiol binds RARs and other proteins.

This result is consistent with our hypothesis that bakuchiol can bind to different target proteins other than that of retinol. Therefore, everything discussed in possible result 1 is also applicable in possible result 3.

Furthermore, since bakuchiol can directly bind to RARs, it is likely that bakuchiol can directly activate the retinol-mediated gene regulation pathway. We recommend conducting further studies to evaluate this possibility.

5 CONCLUSION

Generally, this study tries to identify the molecular target of bakuchiol in human skin cells. However, this study assumes that the chemical coupling between bakuchiol and epoxy-activated agarose beads does not significantly affect the antiaging activity of bakuchiol. If this assumption turns out to be false, the subsequent affinity-chromatography is not applicable, and other experimental approaches should be designed to identify the molecular targets of bakuchiol. If the assumption is true, most likely some protein targets can be isolated and then identified by mass spectroscopy. After the identification of target proteins, we will be able to test the hypothesis that bakuchiol can bind to proteins different than that of retinol (i.e., RARs). After all, this hypothesis is not likely to be false because Chaudhuri and Bojanowski have revealed different gene expression patterns regulated by bakuchiol and retinol (Chaudhuri, Bojanowski 2014).

Further studies should be done to test the hypothesized regulatory pathway of bakuchiol proposed in the Introduction part of this paper. Molecular details involved in this pathway needs to be clarified. Further studies should also examine whether or not the proposed pathway is sufficient to explain the biological activity of bakuchiol. Overall, after a series of studies, the mechanism of action of bakuchiol can be elucidated.

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