HHS Public Access

Author manuscript

Clin Exp Dermatol. Author manuscript; available in PMC 2018 April 01.

Published in final edited form as:

Clin Exp Dermatol. 2017 April ; 42(3): 251–260. doi:10.1111/ced.13024.

Synthesis and activity of the salicylic acid ester of bakuchiol in psoriasis-surrogate keratinocytes and skin substitutes

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Summary

Background—Topical retinoids are effective in retarding skin ageing and restoring homeostasis in skin conditions such as psoriasis. However their adverse effects (AEs), which include irritation (retinoid dermatitis), photosensitivity and teratogenicity, limit their use and patient compliance. Development of retinoid analogues with minimal AEs would allow a broader and more compliant use.

Aim—To synthesise a novel molecule, bakuchiol salicylate (bakusylan), with a modulatory gene expression profile similar to retinoids, using as reference three prescription retinoids: tretinoin, tazarotene and adapalene.

Methods—We hypothesized that because bakuchiol salicylate has a structure entirely different from existing retinoids, there would be at least a partial uncoupling of AEs from the skin normalizing activity of this retinoid. This hypothesis was tested at the transcriptional level in psoriatic cytokine-treated cultures of keratinocytes and organotypic skin substitutes, using DNA microarrays and custom PCR arrays.

Results—Evaluation of the gene expression profile of bakuchiol salicylate revealed elimination of several components of the retinoid-like proinflammatory response and teratogenic signature, without a substantial loss of normalizing potential. A possible mechanism of action, consisting of keratinocyte desensitization to psoriatic cytokine signalling through the inhibition of the signal transducer and regulator of transcription (STAT)1/3/interferon inflammatory signal transduction axis was also identified.

Conclusion—Bipartite materials obtained by merging two skin-active entities with specific, complementary bioactivities, such as bakuchiol and salicylic acid, may yield a new class of functional retinoids.

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Conflict of interest: the authors declare that they have no conflicts of interest.

Introduction

Topical retinoids are effective in delaying skin ageing processes and restoring homeostasis in immune-mediated skin diseases. However, the normalizing effects of retinoids, such as keratinocyte differentiation[1] and inhibition of cytokine-induced gene expression[2–3] carry a price of adverse effects (AEs), which have an impact on tolerance and compliance. Among the most common AEs are irritation and dryness (retinoid dermatitis), which can actually aggravate some symptoms of targeted skin conditions.[4–6] In an attempt to discover retinoid-like compounds with minimal AEs, we screened libraries of natural compounds from plants traditionally used for the treatment of skin conditions for retinoid functionality. This collaborative project yielded one product candidate, a meroterpene and resveratrol structural analogue[7] called bakuchiol.[8,9] In the current study, we report the synthesis of the salicylic acid ester of that molecule, called bakusylan, and its normalizing properties in two *in vitro* psoriasiform-surrogate models.

Psoriasis is characterized by cytokine-triggered alteration of epidermal homeostasis, resulting in a shift from cell differentiation to hyperproliferation and inflammation. T helper (Th)1 and Th17 subsets of T cells play a key role in this process by releasing cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-17A and IL-22, which promote inflammatory response in keratinocytes, leading to further T-cell infiltration and parakeratosis.[10–13] Therefore, our psoriasis-surrogate models to test bakusylan were prepared by treating keratinocyte and skin substitutes with psoriasiform cytokines TNF-α, IL-17A and IL-22.

Methods

Synthesis of bakusylan

Bakuchiol salicylate was synthesized with salicylic acid (Sigma-Aldrich, St Louis, MO, USA) and bakuchiol (Sytheon, Boonton, NJ, USA and Abcam, Cambridge, MA, USA; two different suppliers were used to ensure reproducibility) by the modified Steglich esterification method using dichloromethane as solvent, with dicyclohexylcarbodiimide as condensing agent and 4-*N,N*-dimethylaminopyridine as catalyst. After solvent evaporation and removal of dicyclourea by filtration, the obtained residue was loaded onto a silicon dioxide (high-purity Fluka silica gel, pore size 60 Å, 70–230 mesh) column, and eluted with toluene/cyclohexane (1/1 v/v). The solvent was evaporated under vacuum, and the oily residue was analysed by infrared detection (Satellite FT-IR spectrophotometer; Mattson Instruments, Madison, WI, USA), nuclear magnetic resonance (200 MHz; Gemini; Varian, Palo Alto, CA, USA), diode array spectrophotometry (HP-8452A; Agilent Technologies, Palo Alto, CA, USA) and high-performance liquid chromatography (HPLC) (series 1100; Agilent Technologies) equipped with an injector (Rheodyne® 7725), binary pump, diode array–ultraviolet detection modules, C18 column (Xterra MS Waters; Milford, MA, USA) and ChemStation software. Samples dissolved in ethanol (10 μL) were eluted with 0.1% formic acid/acetonitrile gradient at 0.3 mL/min.

Psoriasis-surrogate human epidermal keratinocytes

Epidermal keratinocyte progenitors from stratum basale (Zen-Bio, RTP, NC, USA) were incubated with CnT-PCT media supplemented with a mixture of IL-17A, IL-22 and TNF-α (100, 100 and 10 ng/mL, respectively; all BioLegend, San Diego, CA, USA), for 24 h, then the test materials were added and the cells incubated in the presence of the cytokines for another 24 h.

Psoriasis-surrogate human epidermal substitutes

Immature epidermal skin substitutes (MatTek, Ashland, MA, USA) were cultured for their final differentiation time (3 days) in media containing IL-17A/IL-22/TNFα (100/100/10 ng/mL, respectively). After the first 24 h of cytokine treatment, the test materials were added and the incubations were pursued in the presence of the cytokines for another 24 h.

PCR arrays and DNA microarrays

At the end of the incubations described above, total RNA was extracted (NucleoSpin II Kit; Macherey-Nagel, Bethlehem, PA, USA). For PCR, reverse transcription was performed using RT^2 First Strand Kit and quantitative PCR was performed (iCycler iQ; BioRad, Hercules, CA, USA), using primers relevant to psoriasis and/or retinoic acid signalling. Gene expression was standardized to the housekeeping gene peptidylprolyl isomerase A (*PPIA*). All PCR reagents were from Qiagen (Germantown, MD, USA).

For the DNA microarray experiment, gene expression was profiled using an array platform (Human Genome U133 Plus 2.0; Affymetrix, Santa Clara, MD, USA), containing > 470 000 transcripts associated with 20 150 human genes. False discovery rate (FDR), was controlled for by adjusting raw *P* values derived from linear models using the Benjamini–Hochberg method.[14] Enrichment of differentially expressed genes with respect to ordered gene lists was performed using the Wilcoxon rank sum test.[15]

Statistical analysis

Statistical analysis of DNA microarray results is described in the relevant section above. Elsewhere, two-tailed (paired) *t*-test was used for estimating statistical significance of differences between comparisons. Differences were considered significant at $P < 0.05$. Experiments were performed in biological duplicates or greater repeats.

Results

Synthesis of bakusylan

The final product [(E)-4-(3,7-dimethyl-3-vinylocta-1,6-dien-1-yl)phenyl 2-hydroxybenzoate)] eluted from the silica column was a light colourless oil. Its identification yielded the following results: IR (KBr): 3226 (ν O-H), 2967, 2924, 2854 (ν C-H), 1691 (ν C=O), 1615, 1583, 1506, 1484 (ν C=C), 1300 (δ O-H), 1193 (ν C-O), 1156, 1066 (δ C-H), 756 (γ C-H) per cm; ^{1}H NMR (200 MHz, $CDCl_3$): 1.23 [singlet (s) 3H, CH_3], 1.24–1.27 (m, 2H, CH_2), 1.60 (s, 3H, CH_3), 1.70 (s, 3H, CH_3), 1.98 (q, 2H, CH_2, J = 8 Hz), 5.00–5.13 (m, 3H, 3CH), 5.84–5.98 [multiplet (m) 1H, CH), 6.20 (d, 1H, CH, J = 14 Hz), 6.37 (d, 1H, CH J = 16 Hz), 6.94–7.07 (m, 2H, ArH), 7.15 (d, 2H, ArH, J = 9Hz), 7.44 (d, 2H, ArH, J = 9

Hz), 7.51–7.55 (m, 1H, ArH), 8.08 [doublet of doublets (dd), 1H, ArH, J_1 = 6 Hz, J_2 = 2H), 10.53 [broad singlet (br s) 1H, OH + D_2O exchangeable) ppm; ^{13}C NMR (200 MHz, $CDCl_3$): δ 18.2, 23.7, 26.2, 30.2, 41.7, 43.2, 112.7, 118.3, 120.0, 122.1 (2C), 125.1, 126.6, 127.6 (2C), 130.8, 131.9, 136.8, 137.0 (2C), 139.2, 146.1, 149.4, 162.7, 169.5 ppm. Elemental analysis was consistent with the expected chemical formula [analytical calculations for $C_{25}H_{28}O_3$ (376.20): C, 79.75; H, 7.50; actual result: C, 79.64; H, 7.39]; purity: >99% (Fig. 1a). λ_{max} was established at 250 nm (Fig. 1b). In HPLC the larger, less polar ester eluted later than bakuchiol (compare Fig.1c with Fig. 1d), as predicted by the nonpolar bonding of salicylate with the parent molecule.

Induction of psoriasis-surrogate phenotypic changes in keratinocyte and organotypic cultures

Various combinations of TNF-α, IL-17A and IL-22 cytokines were tested to induce psoriatic phenotype in human epidermal keratinocyte progenitors (results not shown). The selected concentration (10, 100 or 100 ng/mL, respectively) induced marked shifts in gene expression (Table 1) and cell morphology, consistent with induction of a psoriatic phenotype, after 48 h of exposure [compare Fig. 2a (water control) vs. Fig 2b (cytokine-treated)]. When epidermal skin substitutes were incubated in medium containing the same cytokine cocktail for 48 h, a similar (although not identical) psoriasiform response was observed (Table 1).

Effect of bakusylan on psoriasis-surrogate human epidermal keratinocytes

To determine whether bakusylan can mitigate the effects of psoriasiform cytokines, it was added at 10 μg/mL to cell culture media 24 h after the cytokines were added, and the cells incubated for a further 24 h. Microscopic count of affected cells showed that bakusylan inhibited the cytokine-induced shift in keratinocyte morphology towards the senescent phenotype by about 60% (Fig. 2c vs. Fig. 2b), as quantified by counting enlarged (by > 2.5 times) cells with disorganized cytoskeletons (Fig. 2b, arrows). This effect was comparable with that of 0.1 μg/mL adapalene (Fig. 2e) and better than 1.75 μg/mL tazarotene (Fig. 2d, 40% inhibition). All compounds were assayed at the highest noncytostatic concentrations.

DNA microarrays revealed a retinoid-like effect of bakuchiol salicylate, as illustrated in Fig. 3. This diagram shows the number of probe sets unique to and common between the comparisons with vehicle control for bakusylan, all-*trans* retinoic acid (ATRA) and another antipsoriatic compound (compound C) with no analogy to retinoids. While 39 of the 87 (45%) probe sets that were significantly modulated by bakusylan were also modulated by ATRA, only 13 (17%) of the compound C probes were similarly modulated by ATRA, although both compounds have a transcription-modulatory profile consistent with antipsoriatic activity. This result indicates that bakusylan but not compound C has retinoid-like transcription-modulation activity. Interestingly, this similarity appears limited to a subset of ATRA-modulated genes, which make up only about 20% of the genes modulated by the retinoic acid (39 vs. 197).

To further investigate this selective retinoid functionality, the biological processes most significantly downregulated by ATRA and bakusylan were compared. This comparison identified a desensitization to cytokine stimulus in general, and to type I interferon in

particular, as the most prominent common feature of the two comparators (Table 2), indicating the mechanistic analogy in downregulation of the cytokine-driven inflammatory responses between ATRA and bakusylan. Importantly, this analogy does not extend to morphogenic processes, whose downregulation is associated with the teratogenic effects of retinoids; at least seven morphogenic/developmental pathways were downregulated by ATRA (Table 1) but none by bakusylan. The observed cytokine desensitization seems correlated with the decrease in signal transducer and regulator of transcription 1 (STAT1) ($P < 0.01$) and STAT1-controlled genes, because bakusylan represses genes with a large number of STAT1 [and interferon-stimulated gene factor 3; ISGF3] motifs in the upstream region (Table 3). These results are consistent with other results (Fig. 3, Table 2), and indicate that bakusylan is a selective transcriptional modulator similar to ATRA, but possibly devoid of teratogenic potential.

We next compared bakusylan-downregulated genes with 301 ordered gene lists[16] extracted from microarray datasets deposited in Gene Expression Omnibus (GEO). This comparison identified clinical conditions that similarly led to a decrease (positive association) or an increase (negative association) in the expression of bakusylan-downregulated genes. As shown in Table 4, genes decreased by bakusylan tended to have elevated expression in psoriasis lesions compared with normal skin, whereas genes decreased by bakusylan showed decreased expression in the lesions of patients treated with antipsoriatic drugs. This result supports the concept of bakusylan as a skin-normalizing agent.

Confirmation of the normalizing functionality of bakusylan by PCR arrays in organotypic cultures

The narrow-spectrum, retinol-like, cytokine-desensitizing functionality of bakusylan in psoriasis-surrogate keratinocytes determined by DNA microarrays was further compared with that of three current prescription retinoids (tretinoin, tazarotene and adapalene) in psoriasiform skin substitutes, using PCR arrays (Table 1). The result showed that only bakusylan favourably modulated three important genes dysregulated by psoriasiform cytokines: *STAT3*, *IL-8* and *CXCL3* (*STAT3* is also an important cancer effector).

Discussion

Psoriasis is a chronic immune-mediated disease causing harmful dysregulation of the epidermis, and is a significant health problem as it affects 1–3% of the world population.[17] It is also a good study model for identifying novel functional retinoids, because of its sensitivity to the normalizing effects of this class of compounds.[1–3]

When tested in two *in vitro* psoriasis-surrogate models, bakusylan showed an improved transcription-normalizing profile compared with control retinoids, without a teratogenic retinoid signature. Bakusylan not only downregulated psoriasiform gene expression but also inhibited the expression of IL-8 and CXLC3 in skin substitutes. These proinflammatory cytokines may be responsible for some of irritant AEs of retinoids. Genes whose expression was decreased by bakusylan correlated significantly with the genes whose expression is increased in psoriatic skin and negatively correlated with genes whose expression is

increased by treatment with antipsoriatic drugs. This demonstrates at least partial uncoupling of the pro-irritant side effects of this retinoid from its skin-normalizing effects.

The psoriasis-surrogate keratinocyte model also allowed us to address the mechanism of action of this bakuchiol ester in an inflammatory context. One of the key signatures in psoriasis is induction of interferon (IFN)-mediated pathways associated with STAT1 binding sites.[18,19] Inhibition of interferon expression by ATRA has been reported previously.[20] In the current study, we found that inhibition of type I interferon signal transduction is a common feature between ATRA and bakusylan, and is correlated with a decrease in STAT1 and STAT1-controlled genes. Furthermore, recent analyses suggest that this IFN–STAT1 pathway is activated in a number of different skin diseases and thus the use of bakusylan would not be limited to psoriasis.

Taken together, these results point to a retinoid-like anti-inflammatory potential of bakuchiol salicylate, which would make it suitable for dermatological and skin-care applications. As the effects of retinoids are also exerted on the sebaceous glands, follicular keratinization and certain cancers, it would be interesting to determine whether the functional transcriptional similarity of bakusylan extends in these circumstances as well. Importantly, the narrow activity spectrum of this ester does not include proinflammatory effects or blockage of morphogenic processes, such as cardiovascular and epidermal development, which are common to vitamin A derivatives. AEs are the ‘glass ceiling’ for retinoids, impeding their wider use, and elimination of these AEswould allow a more widespread and compliant utilization of retinoids, unlocking the full functional potential of this class of skin-active compounds. The bipartite materials obtained by merging two skin-active chemicals with complementary bioactivities, such as bakuchiol and salicylic acid, may herald the next generation of functional retinoids.

Acknowledgements

This study was supported by an SBIR grant from the National Institutes of Health, National Institute of Arthritis, Musculoskeletal and Skin Diseases (NIAMS) # 1 R43 AR062935-01A1.

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What's already known about this topic?

- Retinoids are important therapies for psoriasis.
- Teratogenicity and skin irritation are the limiting factors for retinoid utilization in psoriasis.

What does this study add?

- The new compound described here, called bakusylan, is an ester of two skin-active molecules: salicylic acid and the meroterpene bakuchiol
- Using DNA microarrays and PCR arrays, bakusylan (bakuchiol salicylate) was found to be a narrow transcriptional analogue of retinol.
- This narrow retinol functionality appears to at least partially uncouple the AEs of functionality from the therapeutic effects of this retinoid.
- A mechanism of action of bakuchiol salicylate, consisting of keratinocyte desensitization to cytokines through the inhibition of the STAT1/3/interferon inflammatory signalling pathway, is proposed.

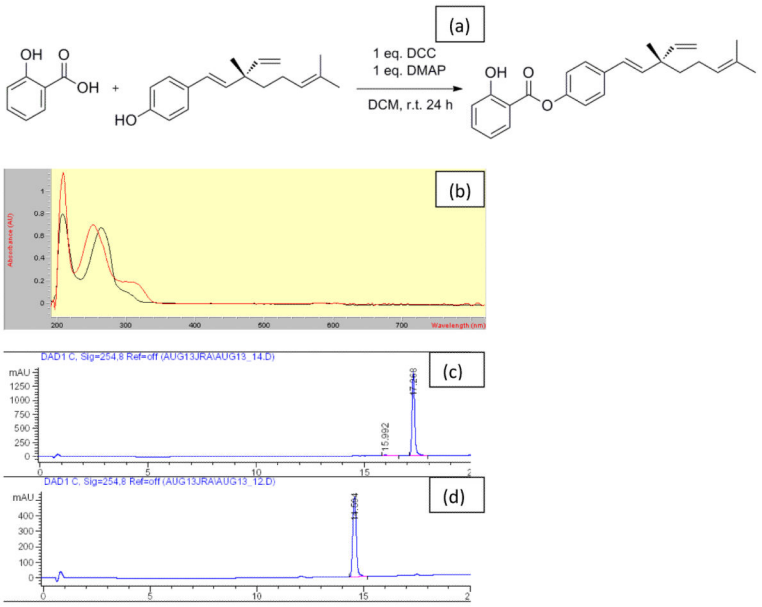

Figure 1.

(a) Synthesis of bakuchiol salicylate (right) from salicylic acid (far left) and bakuchiol (left); (b) spectra of bakusylan and bakuchiol, showing absorption peaks at 250 and 260 nm, respectively; (c,d) reverse-phase high-performance liquid chromatography results for (c) bakusylan and (d) bakuchiol.

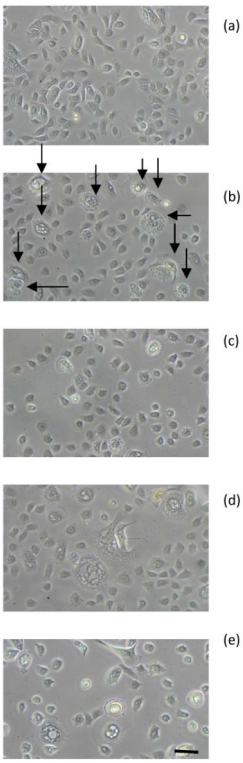

Figure 2.

(a–e) Morphology of human epidermal keratinocyte progenitor either (a) untreated or (b) treated with (b–e) interleukin (IL)-17A/IL-22/tumour necrosis factor (TNF)-α for 48 h. (b) Note the appearance of a subpopulation of cells with 'fried egg' morphology (arrows) in cytokine-treated cells, reminiscent of the proinflammatory senescence-associated secretory phenotype (SASP). (c–e) These gross pathomorphological changes were significantly ($P < 0.05$) reduced with (c) 10 μg/mL bakusylan (approximately thee-fold) (d) 1.75 μg/mL tazarotene (by 40%) and (e) 0.1 μg/mL adapalene (~approximately thee-fold), as determined by 10 direct microscopic counts of senescent cells (arrows). The highest noncytotoxic doses of the test materials were chosen based upon previous cytotoxicity (MTT) experiments. Test materials were added 24 h after the cytokines, then the cells were incubated with the cytokines and test materials for an additional 24 h. Original magnification: × 40; scale bar, 50 μm.

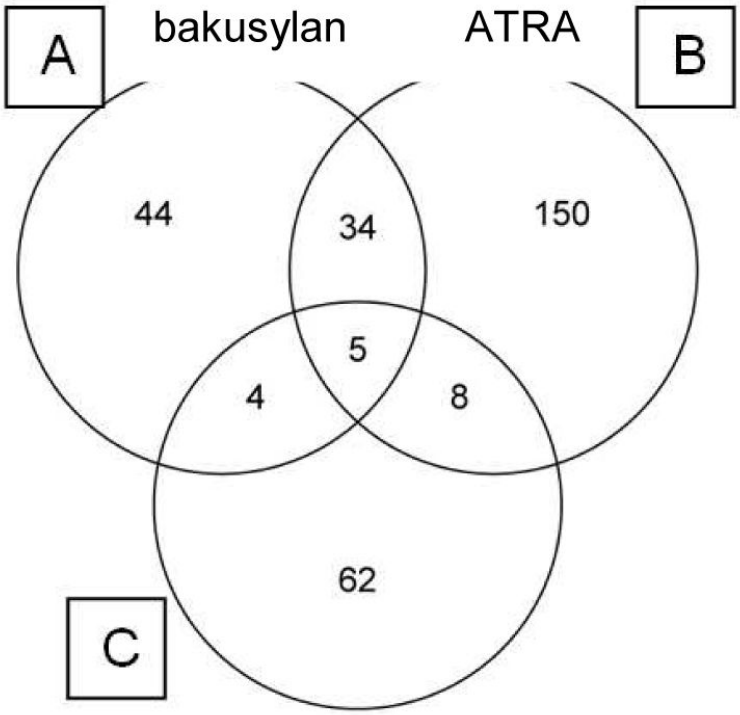

Figure 3.

Bakusylan is a narrow functional analogue of all-*trans* retinoic acid (ATRA) in psoriasiform cytokine-treated keratinocytes. This Venn diagram shows the number of probsets modulated uniquely or jointly by three compounds [(a) bakuchiol salicylate, (b) ATRA, (c) retinoid-unrelated compound with antipsoriatic activity] compared with the vehicle control. Whereas 39 of the 87 probe sets (45%) significantly altered by (a) bakusylan were also modulated by(b) ATRA, only 13 (17%) of the compound C-modulated probes were similarly altered by ATRA, although both molecules have antipsoriatic activity. This result indicates that compound A (bakusylan) is much closer functionally to retinoids than compound C (a fumaric acid derivative). Selected probe sets had a fold change of ≥ 2, with the added constraint that the average expression for one of the two groups was ≥ $2,^{5}$ preventing large fold changes being selected based on small numbers. This statistical analysis of Affymetrix GeneChip data was based on Human U133 Plus 2.0 microarrays that were processed using the IVT Express kit.

Table 1

Effect of bakusylan and control retinoids on the cytokine-induced modulation of the expression of selected genes in keratinocyte progenitor cell cultures and skin substitutes, assessed using custom PCR arrays.

Gene symbol	Cytokines plus:					
	water*	water	BAK	ATRA	ADP	TAZ
S100A7	NC	4.0	NT	NT	NT	NT
DEFB4A	18.9	85.8	−2.0	NC	NC	NC
RARG	−7.5	−3.2	4.1	3.4	NC	3.3
HPGD	−84.5	−27.7	NT	NT	NT	NT
CXCL3	3.3	9.8	−2.1	NC	NC	3.3
IL-8	40.7	67.2	−2.6	3.7	3.4	NC
CTGF	NC	2	NT	NT	NT	NT
SERPINB1	NC	24.0	−7.5	NC	3.3	3.0
HAS3	NC	5.6	NT	NT	NT	NT
KRT10	NC	−2950	NC	−2.5	NC	NC
RARRES1	−2.6	NC	NT	NT	NT	NT
RBP1	−4.6	NC	NT	NT	NT	NT
FLG	NC	−247.3	NT	NT	NT	NT
KRT14	NT	−8	−2.6	−4.3	−2.5	−2.3
PPARG	NT	−3.3	−2.3	NC	NC	6.9
MMP1	NT	1.7	NC	3.3	NC	NC
STAT3	NT	4.6	−2.1	NC	NC	2.5
LOR	NT	−1783	NC	NC	−2.2	−3.3
PTGS2	NT	2.6	NC	NC	NC	−3.9
LAMA3	NT	NC	NC	NC	NC	2.1
LAMC2	NT	NC	4.1	NC	NC	NC
PTEN	NT	−3.2	NC	NC	NC	3.1
AQP3	NT	−9	NC	2.6	NC	3.5

ADP, adapalene; ATRA, all-*trans* retinoic acid; BAK, bakulylan; NC, not changed; NT, not tested; TAZ, tazarotene.

*This column refers to keratinocytes; the remaining columns to skin substitutes. Experiments were performed in duplicate, $P < 0.05$. As shown in column 2, in keratinocytes, these cytokines [interleukin (IL)-17A/IL-22/tumour necrosis factor (TNF)-α] triggered a psoriasis-like phenotype in terms of an increase in expression of *DEFB4A*,[21] *IL-8*[22] and *CXCL3*,[23,24] and a decrease in *HPGD*, which is involved in the catabolism of prostaglandins (prostaglandins play an important role in the pathophysiology of psoriasis;[19] the decrease in *HPGD* by IL-17A/IL-22/TNF- α is a novel finding). *RARG*, *RBP*1 and *RARRES1* are retinoid-responsive genes and were included to monitor mechanistic similarities between the control retinoids and bakusylan. Likewise, in skin substitutes, cytokine-induced gene expression modulation is in agreement with induction of psoriasis-like changes (increase in *DEFB4A*, *CXCL3* and *IL-8*, and decrease of *HPGD*). In addition, there were increases in markers of psoriatic epithelium (*HAS3*, *SERPINB1*, *CTGF*[24–26]) and decreases in markers of epithelial differentiation (*KRT10*, *FLG*). In the psoriasiform skin substitutes, bakusylan lowered the expression of six psoriasis-related genes, including *STAT3* and the proinflammatory, irritation-related cytokines *IL-8* and *CXCL3*, which, by contrast, were unfavourably upregulated by ATRA and adapalene (ADP) (IL-8) and tazarotene (TAZ) (*CXCL3*). Note also the unique downregulation of the proinflammatory senescence marker *SERPINB1* by bakusylan. This profile indicates a possibly greater therapeutic potential of bakuchiol salicylate compared with the control retinoids.

Table 2

Analysis of the GO BP terms most significantly enriched for genes whose expression was decreased by ATRA or bakusylan (DNA microarrays) in psoriasiform cytokine-treated keratinocytes.

BPs decreased by optimal nontoxic dose of:					
ATRA (0.1 μg/mL)			**Bakusylan (10 μg/mL)**		
GOBPID	*P* value	BP Term	GOBPID	*P* value	BP Term
GO:0060337	4.02×10^{-12}	Type I IFN-mediated signalling pathway *	GO:0006695	3.89×10^{-13}	Cholesterol biosynthesis process
GO:0034340	5.82×10^{-12}	Response to type I IFN *	GO:0046165	2.6×10^{-11}	Alcohol biosynthesis process
GO:0051607	6.25×10^{-10}	Defence response to virus	GO:1901615	4.2×10^{-10}	Organic hydroxy compound metabolic process
GO:0043901	1.98×10^{-6}	Negative regulation of multiorganism process	GO:0006694	5.07×10^{-10}	Steroid biosynthesis process
GO:0006955	8.51×10^{-6}	Immune response *	GO:0060337	8.58E-09	Type I IFN-mediated signalling pathway
GO:0045071	1.09×10^{-5}	Negative regulation of viral genome replication	GO:0034340	9.73E-09	Response to type I IFN *
GO:0060333	2.19×10^{-5}	IFN-γ-mediated signalling pathway *	GO:0008299	4.09E-08	Isoprenoid biosynthesis process
GO:0032501	3.02×10^{-5}	Multicellular organismal process	GO:0006639	9.16E-07	Acylglycerol metabolism process
GO:0010033	4.64×10^{-5}	Response to organic substance	GO:0051607	2.09×10^{-6}	Defence response to virus
GO:0035457	8.19×10^{-5}	Cellular response to IFN-alpha *	GO:0046460	2.52×10^{-6}	Neutral lipid biosynthesis process
GO:0051270	8.3×10^{-5}	Regulation of cellular component movement	GO:0051707	8.35×10^{-6}	Response to other organism
GO:0070887	8.46×10^{-5}	Cellular response to chemical stimulus	GO:0019432	2.33×10^{-6}	Triglyceride biosynthesis process
GO:0016525	9.33×10^{-5}	Negative regulation of angiogenesis	GO:0044255	3.93×10^{-5}	Cellular lipid metabolic process
GO:0002688	1.3×10^{-4}	Regulation of leucocyte chemotaxis *	GO:0044710	4.04×10^{-5}	Single-organism metabolism
GO:0034341	1.96×10^{-4}	Response to IFN-γ *	GO:0006955	4.4×10^{-5}	Immune response *
GO:0065007	2.44×10^{-4}	Biological regulation	GO:0010499	1.37×10^{-4}	Proteasomal ubiquitin-independent protein catabolism
GO:0050792	2.49×10^{-4}	Regulation of viral process	GO:0071616	3.55×10^{-4}	Acyl-coenzyme A biosynthesis process
GO:0060585	2.64×10^{-4}	Positive regulation of prostaglandin-endoperoxide synthase activity	GO:0048661	3.93×10^{-4}	Positive regulation of smooth muscle cell proliferation
GO:1901342	2.67×10^{-4}	Regulation of vasculature development†	GO:0009991	4.87×10^{-4}	Response to extracellular stimulus
GO:0072358	2.73×10^{-4}	Cardiovascular system development†	GO:0016042	7.47×10^{-4}	Lipid catabolic process
GO:0048729	3.1×10^{-4}	Tissue morphogenesis†	GO:0045445	8.58×10^{-4}	Myoblast differentiation
GO:0001655	3.83×10^{-4}	Urogenital system development†	GO:0051591	1.07×10^{-3}	Response to cAMP

BPs decreased by optimal nontoxic dose of:					
ATRA (0.1 μg/mL)			**Bakusylan (10 μg/mL)**		
GO:0040012	4.69×10^{-4}	Regulation of locomotion	GO:0046503	1.41×10^{-3}	Glycerolipid catabolic process
GO:0048705	7.47×10^{-4}	Skeletal system morphogenesis†	GO:0019433	1.47×10^{-3}	Triglyceride catabolic process
GO:0000462	7.73×10^{-4}	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA)	GO:0046461	1.47×10^{-3}	Neutral lipid catabolic process
GO:0048646	7.78×10^{-4}	Anatomical structure formation involved in morphogenesis†	GO:0045017	1.771×10^{-3}	Glycerolipid biosynthesis process
GO:0060700	7.83×10^{-4}	Regulation of ribonuclease activity	GO:0043330	1.93×10^{-3}	Response to exogenous double-stranded RNA
GO:0008544	8.03×10^{-4}	Epidermis development†	GO:0035383	1.99×10^{-3}	Thioester metabolic process
GO:0071345	9.1×10^{-4}	Cellular response to cytokine stimulus*	GO:0071345	2.0×10^{-3}	Cellular response to cytokine stimulus*

ATRA, all-*trans* retinoic acid; BP, biological process; GO, Gene Ontology; IFN, interferon; rRNA, ribosomal RNA; SSU, small subunit.

*Four biological processes are common to ATRA and bakusylan, indicating a mechanistic analogy in downregulating the cytokine-driven inflammatory responses between the two compounds. Importantly, this analogy does not extend to morphogenic processes, whose downregulation is associated with teratogenic effects of ATRA; at least seven morphogenic/developmental pathways were downregulated by ATRA† and none by bakuchiol salicylate. These results suggest that bakusylan has a functional similarity to ATRA with possibly no teratogenic potential.

Table 3

Suppression of interferon controlled pathways in cytokine-treated keratinocytes.

Enriched motifs	Motif ID	Esti-mate	SE	Z stat	*P* value	Predicted *STAT1*/*ISGF3* targets downregulated by bakusylan
STAT1: GRAANNGAAAST	STAT_disc3	1.61	0.20	8.01	1.2×10^{-15}	*IFI44L*, *IFIH1*, *DDX60*, *DDX60L*, *EPSTI1*, *MX2*, *RSAD2*, *IFI44*, *ZNFX1*, *TRIM14*, *OAS3*, *MYOCD*, *IFIT1*, *MX1*, *STAT1*, *SP100*, *PNPLA3*, *ITSN1*
STAT1: RGAAANYGAAACT	J9365	2.33	0.31	7.49	7×10^{-14}	*DDX60*, *IFI44L*, *IFI44*, *PARP9*, *TRIM14*, *IFIH1*, *IFIT1*, *STAT1*, *DDX60L*, *EPSTI1*, *MX2*
Isgf3g: RAAWCGAAACT	UP00074	2.28	0.32	7.02	2.2×10^{-12}	*DDX60L*, *DDX58*, *RSAD2*, *IFI44L*, *TRIM14*, *OAS3*, *DDX60*, *MX1*, *STAT1*, *EPSTI1*

Bakusylan suppressed interferon-controlled pathways in psoriasiform cytokine-treated keratinocytes by repressing multiple genes (right column) with significant enrichment/over-representation of *STAT1* (and *ISGF3*) motifs (first column) in their upstream region (5 kb). Induction of interferon-mediated pathways associated with STAT1 binding sites is a robust signature in psoriasis lesions.[19,20] Enrichment of motifs was calculated using semiparametric generalized additive logistic models.[19]

Table 4

GEO database microarray experiments leading to increased or decreased expression of bakusylan-downregulated genes.

GEO accession (experiment ID)	Gene signature from the GEO database for the given set of experiments	Correlation with genes downregulated by bakusylan
GSE2737, GSE6710, GSE11903, GSE13355, GSE26866, GSE30999, GSE14905, GSE26866, GSE26866	Genes upregulated in psoriatic vs normal tissue	Negative *
GSE7553, GSE4587, GSE4587	Genes upregulated in cancerous vs normal tissue	Negative *
GSE32620, GSE32620, GSE24767, GSE20297, GSE7216, GSE36287, GSE1132, GSE7216, GSE12109, GSE36287, GSE2489, GSE32975, GSE36387, GSE20297, GSE9120, GSE25400, GSE7216, GSE20297, GSE24767, GSE28158, GSE37361, GSE37361, GSE24767, GSE32407, GSE32407, GSE24873	Genes upregulated in IL1-, IL17-, IL22-, TNF- and/or IFN-treated tissues compared with untreated tissues	Negative †
GSE16161, GSE32924, GSE5667, GSE5667	Genes upregulated in atopic skin vs normal skin	Negative
GSE26487, GSE26523, GSE2822, GSE11903, GSE31652	Genes upregulated by antipsoriatic drugs (LY2439821, etanercept, efalizumab, dexamethasone) vs untreated psoriatic tissue	Positive ‡
GSE22298, GSE11792, GSE10433	Genes upregulated in tissues treated vs retinoids	Positive
GSE30768	Genes upregulated in efalizumab-treated psoriatic skin after relapse vs no relapse	Negative

IFN, interferon; IL, interleukin, TNF, tumour necrosis factor; GEO, Gene Expression Omnibus.

* Genes upregulated in psoriatic skin in the cited GEO experiments that were downregulated by bakusylan;

† genes upregulated by tissue treatment with IL1-, IL17-, IL22-, TNF- and/or IFN in the cited GEO experiments that were downregulated by bakusylan;

‡ genes downregulated in the skin of psoriatic patients treated with antipsoriatic drugs in the cited GEO experiments, which were also downregulated by bakusylan. We screened ordered gene lists derived from GEO to identify experiments with gene signatures that correlated with our own microarray data. Experiments with increased expression of bakusylan-downregulated genes (negative association) and decreased expression of bakusylan-downregulated genes (positive association) were identified. The GEO series accession IDs listed denote experiments showing significant association ($P < 0.01$; false discovery rate < 0.05). To obtain the enrichment statistic value we used equation (8) from Philippakis *et al.*[15]