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Abbreviations used: HB-EGF: heparin-binding epidermal growth factor-like growth factor, RA: all-*trans* retinoic acid

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Abstract

Background: All-*trans* retinoic acid (RA) is known to regulate keratinocyte proliferation and differentiation and retinoids are used as therapeutic agents in certain dermatological disorders (e.g. psoriasis, acne). Epidermal expression of the heparin-binding EGF-like growth factor (HB-EGF) is induced by RA treatment and HB-EGF is responsible for RA-mediated epidermal hyperplasia *in vivo*. RA also induces HB-EGF expression in cultured keratinocytes and alters their differentiating phenotype. R115866 is a specific inhibitor of the cytochrome P450 isoform CYP26, which is involved in the metabolic inactivation pathway of RA. Thereby, R115866 is thought to be able to increase the intracellular levels of endogenous RA.

Objectives: To determine whether R115866 potentiates the effect of RA at low concentrations on keratinocytes.

Methods: We analyzed HB-EGF, involucrin and keratin 10 mRNA and protein levels in autocrine human keratinocyte cultures incubated for 18h with RA or R115866 alone and with RA and R115866 combinations.

Results: RA induces HB-EGF and involucrin expression in a concentration-dependent manner, whereas it inhibits keratin 10. R115866 alone has no effect on the expression of these genes. However when R115866 is combined with low concentrations of RA, we observe inductions of the expression of HB-EGF and involucrin.

Conclusion: These results strongly suggest that R115866 potentiates the effects of RA on epidermal keratinocytes when RA is present at low concentrations.

215 words

Introduction

All-*trans* retinoic acid (RA) is an indispensable natural regulator of epidermal proliferation and differentiation and retinoids are used as therapeutic agents for the treatment of dermatological disorders such as psoriasis, acne or skin aging [1]. These compounds exert their biological activity through binding with transcription-regulatory factors, the retinoic acid receptors (RARα, β and γ) and the retinoic X receptors (RXR α, β and γ). Only RARα, RARγ, RXRα and RXRβ are expressed in the epidermis [2]. Although studies of the effects of RA on epidermal differentiation have sometimes produced controversial results when obtained *in vivo* or *in vitro* [1], it appeared that inducing or inhibiting effects can depend also on the differentiation markers analyzed [3]. Particularly in autocrine high cell density cultures of epidermal keratinocytes, RA markedly inhibits the mRNA and protein expression of the suprabasal keratin 10 (K10), an early marker of epidermal differentiation, whereas RA induces both mRNA and protein levels of involucrin, a later marker especially expressed in upper spinous and granular layers in the differentiated normal epidermis *in vivo* [3]. Besides effects on epidermal differentiation, RA is also able to induce keratinocyte proliferation and thus epidermal hyperplasia. Recent studies have shown that this phenomenon is due to an induction by RA of the heparin-binding EGF-like growth factor (HB-EGF) [4, 5], a potent stimulator of keratinocyte proliferation and migration which belongs to the epidermal growth factor (EGF) family and which can be induced by stress conditions in keratinocytes [6]. Because RA has profound effects on epidermal growth and differentiation, there is a tight homeostatic control of its level in tissues like the epidermis due to RA inactivation by cytochrome P450 (CYP) isozyme system(s). The isozyme CYP26 is particularly involved as RA is the only recognized substrate of CYP26 [7]. Recently, CYP26 was detected in human skin, principally in the epidermal basal layer, and RA was shown to be an inducer of CYP26 expression in normal cultured keratinocytes [8].

R115866 (talarozole or Rambazole™; Barrier Therapeutics, Geel, Belgium) is a specific inhibitor of CYP26 and induces, when delivered *in vivo*, an increase in tissue levels of endogenous RA by blocking RA catabolism [9, 10]. Thus, R115866 increases RA in plasma and skin, exerting cutaneous effects that have also been described with retinoids [9]. Recently, clinical trials have started to demonstrate the efficacy of R115866 in the treatment of psoriasis and acne [10-12]. R115866 (third generation retinoic acid metabolism blocking agents or RAMBAs) is a nanomolar (IC_{50}=4nM) inhibitor of CYP26-dependent RA conversion and is

about three orders of magnitude more powerful than liarozole ($IC_{50}=3\mu M$) (first RAMBAs generation) which lacked CYP isozyme specificity [9].

In this study, we have taken advantage of the unique environmental conditions obtained during autocrine growth of epidermal keratinocytes because in such conditions the expression of epidermal differentiation markers has been thoroughly characterized in total absence of serum or of other sources of exogenous factors, and because this model is thus also totally devoid of any source of vitamin A [3,13]. We analyzed the effects of RA, R115866, and RA in combination with R115866 in autocrine cultures of normal human epidermal keratinocytes. All experiments described here were performed at the cell confluent state which is characterized by cell growth arrest and initiation of the commitment towards differentiation. These conditions are very likely uniquely suited for the identification of subtle regulations of differentiation. We focused on HB-EGF, involucrin and keratin 10 gene expression and observed that, contrarily to RA, R115866 alone is unable to induce any modification in the expression of those genes. However, when R115866 is combined with RA at low concentration (10^{-9} M), the expression of both HB-EGF and involucrin is increased, whereas Keratin 10 gene expression is inhibited, very similarly to the effects obtained with a ten-fold higher concentration of RA (10^{-8} M).

Together, our data show that the CYP26 inhibitor R115866 potentiates the effect of nanomolar concentrations of RA on the differentiation process in epidermal keratinocytes.

Materials and Methods

Antibodies and chemicals

Rabbit polyclonal anti-ERK1/2 antibody was obtained from Upstate (Millipore, Brussels, Belgium) and goat anti-human HB-EGF antibody was from R&D Biosciences (Abingdon, UK). Rabbit anti-human involucrin antibody was purchased from Harbor Bio-products (Boechout, Belgium). Mouse anti-human keratin10 antibody was from Dako (Heverlee, Belgium). Rabbit anti-human EGFR antibody was purchased from Cell Signaling (Leiden, The Netherlands) and rabbit anti-human phosphor-EGFR Tyr1173 antibody was from Biosource (Nivelles, Belgium). Mouse anti-human HER2 and rabbit anti-human phosphor-HER2 came from Cell Signaling (Leiden, The Netherlands). Retinoic acid was purchased from Sigma-Aldrich (Bornem, Belgium) and R115866 is from Barrier Therapeutics (Geel,

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Belgium). Keratinocyte growth medium (KGM2) came from Lonza (Verviers, Belgium). Keratinocyte complete culture medium (Epilife and HKGS) and keratinocyte autocrine medium (Epilife) were from Cascade Biologics (Manfield, UK). AEC, glycergel and horseradish peroxydase (HRP)-conjugated anti-rabbit were obtained from Dako (Heverlee, Belgium).

Cell culture and treatment

Normal human keratinocytes were isolated by the trypsin float technique [14] from adult skin samples obtained at plastic surgery (Dr Bienfait, Clinique St. Luc, Namur-Bouge, Belgium). Primary cultures were initiated in complete keratinocyte growth medium (KGM2). Proliferating primary cultures were trypsinized and keratinocytes were plated into secondary cultures at $6x10^3$ cells per cm^2 in complete culture medium (Epilife containing HKGS). When the cells covered approximately 40% of the culture substratum, keratinocytes were switched into an autocrine medium (Epilife alone) which does not contain any growth factor or serum. In such conditions, keratinocytes proliferate autonomously until the confluence of the culture is reached concomitantly with cell growth arrest [3,13,14]. All experiments were performed exactly at confluence. Autocrine keratinocyte cultures were treated 18 h with RA (concentrations ranging from 10^{-9} to 10^{-6} M) or R115866 (concentrations ranging from 10^{-9} to 10^{-6} M) before analysis.

Stratified cultures of keratinocytes

For the production of reconstructed human epidermis (RHE), we used our previous published method [15]. Briefly, second- to third-passage proliferating keratinocytes were collected and plated at high density on polycarbonate cultures inserts with 0.4 μm diameter pore size (Millipore) in Epilife medium containing HKGS and 1.5 mM $CaCl_2$. After 24h, the keratinocytes were exposed to the air-liquid interface and we used a medium Epilife containing HKGS, 1.5 mM calcium, 50 μg/ml vitamin C and 10ng/ml KGF to feed the cells from the bottom of the polycarbonate filter. Every 2 days, this medium was renewed and the RHE was treated after 11 days with RA (concentrations ranging from 10^{-9} to 10^{-6}M) or R115866 (concentrations ranging from 10^{-9} to 10^{-6}M) during 2 days.

Total RNA isolation and Real-time PCR

Total RNA were extracted by the *RNeasy kit* (Qiagen, Hilden, Germany)) and then reverse transcribed into cDNA by the *Super Script II RNase H-reverse transcriptase kit* (Invitrogen, Merelbeke, Belgium). Power SYBR Green PCR Master Mix (Applied Biosystems, Lennik, Belgium) was used for the Real-Time PCR. Genes were normalized to the gene 36B4 [16]. The primers sense and anti-sense (300nM, Sigma-Aldrich, Bornem, Belgium) sequences were: for 36B4 sense 5'-ATCAACGGGTACAAACGAGTC-3', 36B4 anti-sense 5'-CAGATGGATCAGCCAAGAAGG-3', HB-EGF sense 5'-TGGCCCTCCACTCCTCATC-3', HB-EGF anti-sense 5'-GGGTCACAGAACCATCCTAGCT-3', for involucrin sense 5'-TGAAACAGCCAACTCCAC-3', involucrin anti-sense 5'-TTCCTCTTGCTTTGATGGG-3', keratin 10 sense 5'AATCAGATTCTCAACCTAACAAC-3' and keratin 10 anti-sense 5'-CTCATCCAGCACCCTACG-3'.

Protein extraction and Western blotting

Before lysis, keratinocytes were washed with PBS and then scraped into twice concentrated Laemmli sample buffer (Tris-HCl 62.5mM, SDS 2%, glycerol 8.7%, bromophenol blue 0.05%, DTT 0.2%). Thereafter, the proteins extracted were analyzed by SDS-polyacryamide gel electrophoresis (PAGE) and transferred onto PVDF membranes (GE Healthcare Biosciences, Uppsala, Sweden). The membranes were blocked with PBS/Tween20 (0.1%) containing 5% powdered milk, before incubating with specific primary antibodies. After three washing steps, the membranes were incubated with HRP-conjugated secondary antibodies (Dako, Hervelee, Belgium). Finally, chemoluminescent detection was realized by using BM Chemoluminescence Blotting Substrate (Roche Diagnostics, Mannheim, Germany). The detection of ERK protein as described previously [17] is used as a loading control.

Measurement of the release of HB-EGF

Release of HB-EGF in the culture medium was quantified by ELISA using the DuoSet ELISA human HB-EGF kit from R&D systems (Abingdon, UK). The optical density was determined at 540 nm (wavelength correction at 450 nm) using a microplate reader (Molecular devices, Sunnyvale, CA).

Immunohistochemical labelling of reconstructed human epidermis sections

After the respective treatments, the RHE were washed with PBS, separated from the inserts and immediately embedded in optimal cutting temperature (OCT) compound. Frozen sections (6 μm thick) of RHE were made with a cryostat and fixed in 4% paraformaldehyde for 15 minutes followed by washes in 0.1 M glycine. In order to inhibit endogenous peroxidases, every tissue section was incubated in 3% H_2O_2 for 10 minutes, then blocked and permeabilized in PBS containing 0.1% BSA and 0.02% Triton X-100. After the blocking step, all sections were incubated for 1 hour with appropriate primary antibody diluted in PBS/BSA/Triton X-100 (mouse anti-human K10 antibody). All slides were then washed with PBS and for keratin 10 immunolabelling the sections were then incubated with HRP-secondary antibody (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Detection of HRP was performed with AEC Substrate-Chromogen and counterstaining with hemalun was done for 5 minutes. All slides were mounted in glycergel for observation in an Olympus AX70 microscope.

Results

RA treatment dose-dependently alters HB-EGF, involucrin and keratin 10 gene expression whereas R115866 does not

Autocrine cultures of human epidermal keratinocytes were studied at confluence when they initiate differentiation [3] and treated for 18h with either RA or R115866 at concentrations ranging from 10^{-9}M to 10^{-6}M. Observation of the cultures by phase-contrast microscopy revealed no alteration in morphology of the keratinocytes treated with each of both compounds at all the tested concentrations (data not shown). We then analyzed by quantitative RT-PCR the mRNA expression of HB-EGF, as well as of involucrin and keratin 10, respectively late and early markers of epidermal differentiation. Our results confirm that RA treatment upregulates dose-dependently HB-EGF [4] and involucrin gene expression, whereas keratin 10 is downregulated (Fig 1) [3]. In parallel, we observed that in the absence of RA, after 18h of treatment, the CYP26 inhibitor R115866 has no effect by itself on HB-EGF, involucrin or keratin 10 gene expression in confluent cultured keratinocytes (Fig 1).

These effects of RA or R115866 alone were also investigated at the protein level. Protein extracts from autocrine confluent keratinocytes in culture were analyzed by Western blot. As shown in Figure 2a, RA dose-dependently induces HB-EGF protein expression, whereas no

effect of treatment with R115866 at any of the tested concentrations is observed on HB-EGF protein expression. The results of this representative experiment correlate properly with the HB-EGF mRNA expression by keratinocytes shown in Figure 1 and reveal again that R115866 has no effect on HB-EGF per se in culture conditions where RA is absent.

HB-EGF is synthesized as a transmembrane protein that can be cleaved enzymatically to release a soluble growth factor (mature HB-EGF) which can bind and activate the EGF receptor (EGFR) or indirectly activate the EGF receptor 2 (HER2). Thus, in order to determine if HB-EGF is released in our model, we measured HB-EGF by ELISA in the culture media after treatments. We found some release of HB-EGF in good accordance with its production (Fig 2b). Indeed, 10^{-6}M RA induces the highest release of HB-EGF (75 pg/ml). In order to explore whether the release of HB-EGF is able to activate receptors, we analyzed after 18h of RA or R115866 treatment the tyrosine phosphorylation of EGFR and HER2 (Fig 3). The results suggest that the release of HB-EGF is probably too weak in order to stimulate detectable phosphorylation of both receptors. However, De Potter et al. have shown that the activation of HER2 in keratinocytes depends on the state of differentiation [18], and found that heregulin can activate HER2, probably through HER3, two days after reaching confluence. Thus, in our model studied at confluence, the activation of HER2 (and HER3, data not shown) cannot be observed in the cultured keratinocytes, maybe because these cells are not yet in a receptive phenotype in our assay.

We also used Western blot analysis in order to confirm the effect of RA or R115866 on keratin 10 and involucrin differentiation markers: RA treatment significantly upregulated involucrin and reduced keratin 10 protein expression in perfect accordance with the mRNA expression (Fig 3). R115866 alone has no effect on the expression of these proteins.

R115866 combined with RA alters the RA-induced changes in gene expression of epidermal keratinocytes

R115866 acts as an inhibitor of the CYP26-mediated metabolism of RA and therefore R115866 treatment enhances the intracellular levels of endogenous RA [9]. This enhancement is impossible in autocrine culture conditions since, in absence of serum, there is no endogenous RA in the culture medium. This can explain the absence of effect of R115866 when the compound is added alone as in Figure 1. Thus, in order to investigate the effects of a combination of R115866 with RA at low concentrations, R115866 at concentrations ranging

from 10^{-9}M up to 10^{-6}M was added to the culture medium containing RA at low concentrations (10^{-9}M or 10^{-8}M). Then, we analyzed the mRNA levels as above by quantitative RT-PCR. Figure 4a illustrates the HB-EGF, involucrin and keratin 10 mRNA expression patterns after 18h of exposure of autocrine cultures of human keratinocytes with R115866 combined with 10^{-9}M RA. Compared with RA alone, a significant increase in the expression of both HB-EGF and involucrin is observed when R115866 concentration increases (Fig 4a). Since R115866 prevents the breakdown of RA, sustained effective cellular concentrations of RA are likely responsible for the enhanced effects due to the combinations of RA with R115866. Accordingly, the downregulation of keratin 10 gene expression after a treatment of R115866 (10^{-9}M) combined with 10^{-9}M RA suggests that the intracellular concentration of RA remains high enough in order to inhibit additionally the keratin 10 mRNA expression (Fig 4a).

Similarly, a treatment with R115866 combined with RA at 10^{-8}M was also performed (Fig 4b). Although the highest R115866 concentration applied (10^{-6}M) induces a significant enhancement in the mRNA expression level for HB-EGF, the lower concentrations do not induce a statistically significant enhancement. This is probably due to the substantial induction already produced by 10^{-8}M RA in the expression of HB-EGF at this concentration. Similarly, R115866 combined with 10^{-8}M RA does not induce further involucrin gene expression. Surprisingly, one can observe an increase due to R115866, but the level remains below the level induced by 10^{-8}M RA alone. Also, as RA at this concentration highly inhibits keratin 10 mRNA expression, the addition of R115866 is unable to inhibit further the expression level of this marker.

The protein expression of HB-EGF in cellular extracts prepared from keratinocytes treated with the combination of R115866 and RA was analyzed by Western blot and a representative experiment is illustrated in Figure 5a. R115866 at increasing concentrations combined with 10^{-9}M RA upregulates the HB-EGF protein expression (Fig 5a), in perfect accordance with the HB-EGF mRNA analysis performed in the same conditions (Fig 4a). However, when studying keratinocytes treated with 10^{-8}M RA, a concentration that already strongly induces the expression of HB-EGF (Fig 2a), the combination with R115866 at 10^{-9}M or 10^{-8}M concentrations does not increase this expression, contrarily to the highest concentrations (10^{-7}M and 10^{-6}M) of R115866 which seem to increase the detection of HB-EGF.

To investigate the shedding of HB-EGF after R115866 combined with RA, we collected the culture media and measured HB-EGF by ELISA. It is interesting to notice that the profile of

HB-EGF release is parallel with its protein production (Fig 5b). The ELISA analysis shows that 10^{-9}M RA induces a weak release of HB-EGF (35 pg/ml), and when R115866 is combined with 10^{-9}M RA, we observed an upregulation of HB-EGF concentration in the medium (65 pg/ml). On the other hand, we observed as in Figure 5a that 10^{-9}M and 10^{-8}M R115866 combined with 10^{-8}M RA treatment does not increase the shedding of HB-EGF (Fig 5b).

Again, the shedding of HB-EGF is insufficient to induce the phosphorylation of EGFR and HER2 (Fig 6). We found also that keratin 10 is downregulated after 10^{-9} M RA treatment compared to the control. We observed that this expression decreases with the addition of R115866 at the highest concentrations (10^{-7}M and 10^{-6}M). Similarly, when we compared the RA treatment (10^{-8}M) and the combination of R155866 and 10^{-8}M RA, we observed that R115866 strongly downregulates the expression of keratin 10 (Fig 6). After 10^{-8} RA treatment, the involucrin expression is mainly induced and thus, as learned from RT-PCR analysis, no upregulation happens when R115866 is added (Fig 6).

Altogether, our results demonstrate that, during 18h incubation, R115866 has no effect on human epidermal keratinocytes in total absence of RA. Rather they show that R115866 potentiates the effect of RA at a concentration that is too low (10^{-9}M) to strongly alter the gene expression of these cells. This potentiation effect was also observed when R115866 was combined with RA at 10^{-10}M concentration (data not shown). When RA is present at a concentration high enough to induce by itself effects on keratinocytes (i.e. 10^{-8}M), any potentiation by R115866 becomes more and more difficult to detect.

The effects of RA and R115866 treatments can be extended to reconstructed human epidermis (RHE).

Since the discrepancy between retinoids action in human monolayers cultures and human skin in vivo is well known [1], we wondered whether similar effects of RA or R115866 and the combination between R115866 and RA could be observed within the reconstructed human epidermis (RHE) [15].

The immunolabelling of keratin 10 revealed that in suprabasal cell layers, mainly just above the basal keratinocytes layer (Fig 7a, arrows), keratin 10 is strongly expressed in the Ctl and DMSO conditions or after R115866 (10^{-6}M) treatment (Fig 7a). After RA treatment, we

observed that keratin 10 expression is reduced in this layer mainly at a 10^{-6}M RA. Keratin 10 was also reduced after R115866 (10^{-6}M) combined with RA (10^{-9} M) (Fig 7a).

The immunolabelling of involucrin was also performed, but revealed no variation between the different conditions (data not shown). Of course, after 7 days of culture, keratinocytes are well differentiated in the RHE model and have already expressed involucrin before the treatment. Thus, it seems impossible to show any variation in involucrin protein expression between the different culture conditions.

These variations between the results obtained with the RHE and the autocrine culture of keratinocytes might likely be explained also by other differences in culture conditions: in the RHE model, although the culture is serum-free, the medium contains growth factors and vitamin C which are necessary in order to obtain a well differentiated stratified epithelium. Particularly, growth factors can interfere with expression of differentiation markers [13]. Thus, our data illustrate that, in order to identify regulation of epidermal differentiation, a keratinocytes monolayer model in autocrine conditions allows a very precise analysis of differentiation markers gene expression. Our data also suggest that fine regulations are more difficult to observe in a more complex model of epidermal differentiation.

Discussion

Retinoids are used as therapy for many skin diseases such as psoriasis and acne. They are important regulators of epidermal proliferation and differentiation. However the effects of RA obtained *in vivo* or *in vitro* on epidermal proliferation or differentiation are sometimes contradictory [1], but it appeared that it can depend on the differentiation markers analyzed and on the culture conditions [3, 19]. Here, we first confirm that addition of RA to the autocrine culture medium of confluent keratinocyte cultures induces a diminution of keratin 10 mRNA expression, whereas the expression of involucrin is upregulated [3]. We further confirm that the epidermal expression of HB-EGF is also highly induced by the treatment of these cells with RA [4].

The cellular metabolic inactivation of RA (which is initiated by hydroxylation) is carried out mainly by an isozyme of the cytochrome P450 system, specifically CYP26, and RA is apparently the main substrate for CYP26 [9]. R115866 is a highly specific and potent CYP26 inhibitor, thus inhibiting in particular the catabolism of RA [9]. Therefore, R115866 can enhance the tissue levels of endogenous RA and thereby is able to produce biological effects

similar to those of retinoids in several experimental assays [9], but also in clinical treatments of psoriasis and acne [10,12].

The unique autocrine culture model of keratinocytes, which means culture in a chemically-defined medium without addition of any peptide exogenous factor or serum (and consequently without RA), allows a precise study of keratinocyte responses to R115866 either in the absence or presence of RA, added at well-defined concentrations. In the present study, we have shown that addition of R115866 alone to the medium of confluent keratinocyte cultures performed in autocrine conditions has no effect on HB-EGF, involucrin and keratin 10 gene expressions in these cells. However, R115866 combined with RA at 10^{-9}M concentration increases HB-EGF and involucrin expression, and concomitantly decreases keratin 10 expression (Figs 4 and 5). These results suggest intracellular elevation of RA concentration when compared with cultures treated with RA alone (Figs 1 and 2). When R115866 at 10^{-6}M is combined with 10^{-8}M RA, the RA-induced upregulation of HB-EGF is further upregulated, but no significant alteration of the RA effect on involucrin and keratin 10 gene expression is observed, probably due to the significant effects already obtained with RA alone at the 10^{-8}M concentration.

We have observed that RA treatment or treatment with R115866 combined with RA induce HB-EGF released (Figs 2 and 5), however EGFR and HER2 were not phosphorylated in our experimental conditions. This can be due to the internalization of the receptor (and degradation) induced upon ligand-binding, or alternatively this can be the consequence that the EGFR activation does not last enough in order to be still detectable after 18h of treatment. Indeed, we have clearly observed that, after 1h of RA treatment, the EGFR is activated (data not shown).

We have also analyzed the effects of RA or R115866, as well as the effects of R115866 combined with RA, on loricrin and filagrin gene expression and we found no alteration of theses genes expression (data not shown). This is not surprising since all experiments have been performed at confluence state, which is characterized by cell growth-arrest and commitment to early differentiation, whereas loricrin and filagrin are only expressed in the granular and cornified layer in the epidermis. Although treatment with RA and treatment with the combination of R115866 with retinoic acid have large effects on differentiation (keratin 10 and involucrin), consequences depend on the differentiation markers analyzed in the study. In conclusion, our results clearly show that during an 18h incubation R115866 exerts effects on the expression of specific differentiation markers in epidermal keratinocytes by its ability to prevent the degradation of RA. Indeed, R115866 enhances the effects of low concentrations

of RA, producing effects similar to those obtained at higher concentrations of RA. This demonstration that R115866 is acting on keratinocytes through RA may explain, at least partially, recent clinical data which have revealed the efficacy of R115866 in the treatment of psoriasis and acne [10,12].

The effects of RA, R115866 and the combination between RA and R115866 on a model of reconstructed human epidermis (RHE) have been investigated. The immunolabelling of keratin 10 shows similar results compared to the results obtained with keratinocyte monolayers. The effects producing a decreased expression of keratin 10 is localized to the immediate suprabasal layer, where the neo-synthesis of keratin 10 is strongly induced upwardly moving keratinocytes. However, contrarily to Hsia et al. [20] who show a complete decrease of the keratin 10 expression in the RHE model treated with topical application of retinoids for 3 days, we do not observe such a strong disappearance of the keratin 10 protein.

It appears that the model used for the study of the effects of RA or R115866 on markers of differentiation can produce particular data due to the state of differentiation in the model.

Thus, in order to identify regulation of differentiation, a monolayer model in autocrine conditions allows a precise analysis of differentiation markers gene expression at a crucial phenotypical state. Our results obtained with the RHE suggest that fine regulations are more difficult to observe in a more complex model.

Altogether, our results suggest that the autocrine culture model of keratinocytes is very adequate for the precise study of RA effects on this cell type. They also show that R115866, a specific inhibitor of CYP26 isozyme, has no effect on epidermal keratinocytes in absence of RA, but when RA is added at low concentrations R115866 is apparently able to maintain the endogenous concentration of this retinoid at a relevant level, thereby resulting into an enhanced retinoid activity. Thus, R115866 potentiates the effects of RA on normal epidermal keratinocytes.

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Conflict of interest

PS is a former employee of Barrier Therapeutics.

Figures

Retinoic acid

R115866 (Rambazole)

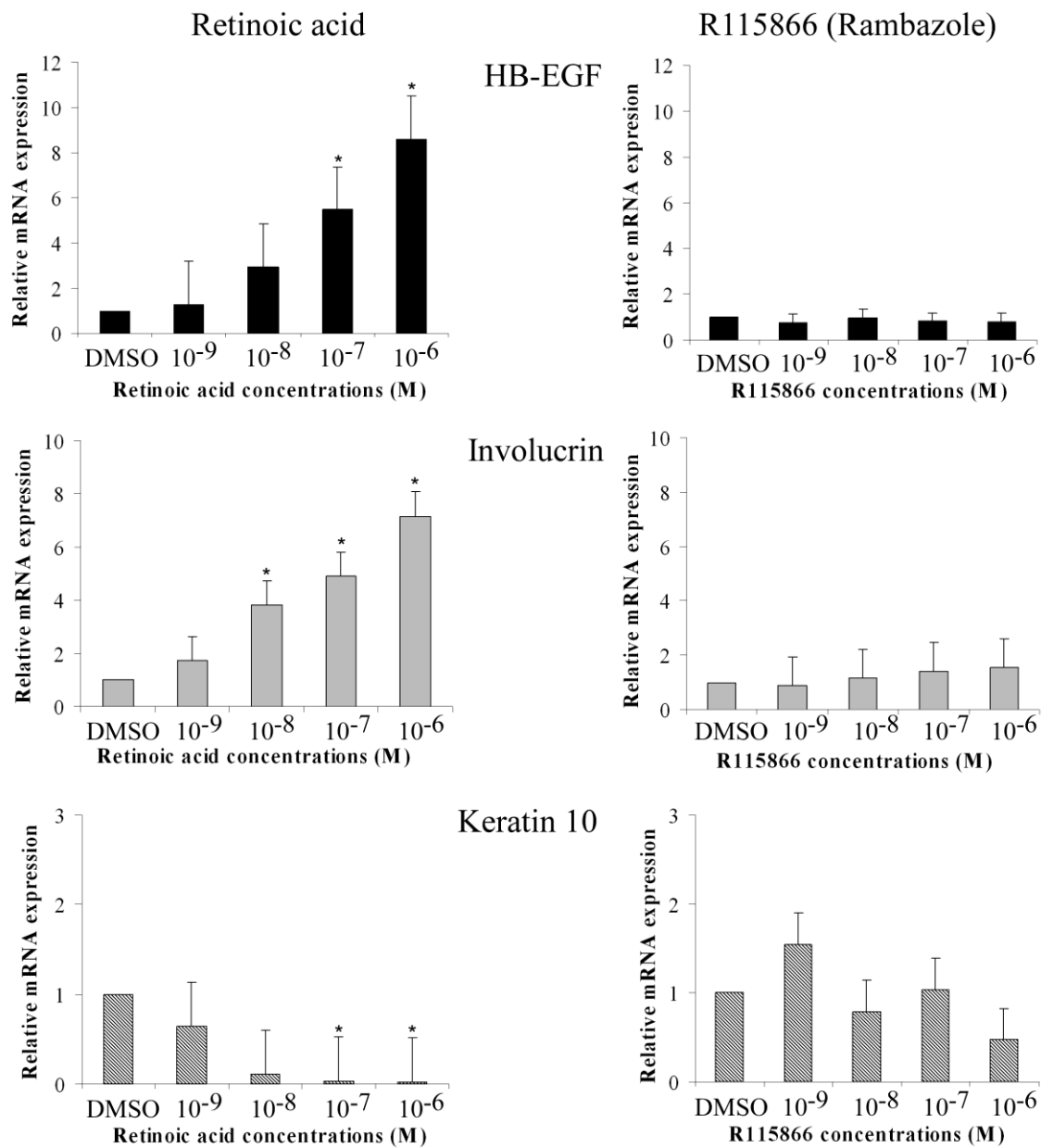**HB-EGF**

Involucrin

Keratin 10

Fig 1: RA treatment alters HB-EGF, involucrin and keratin 10 gene expression, whereas R115866 has no effect.

Confluent keratinocyte cultures were treated for 18h with increasing RA or R115866 concentrations ranging from 10^{-9}M to 10^{-6}M. Total RNA were extracted and reverse-transcribed into cDNA before analysis by Real-Time PCR in order to determine *HB-EGF* (■),

involucrin (▪) or *keratin 10* (▨) gene expression (normalized to *36B4*). Data represent three independent experiments and were analyzed by ANOVA I after testing the homogeneity of variance (Bartlett). Post-hoc comparisons were performed by pairwise Scheffe's test. Error bars represent ± 2 SEM (Standard Error of the Mean) with Student's correction for variance estimation. To ensure homosedasticity, values for keratin 10 expression (x) were replaced by their logarithmic values (log (x)).
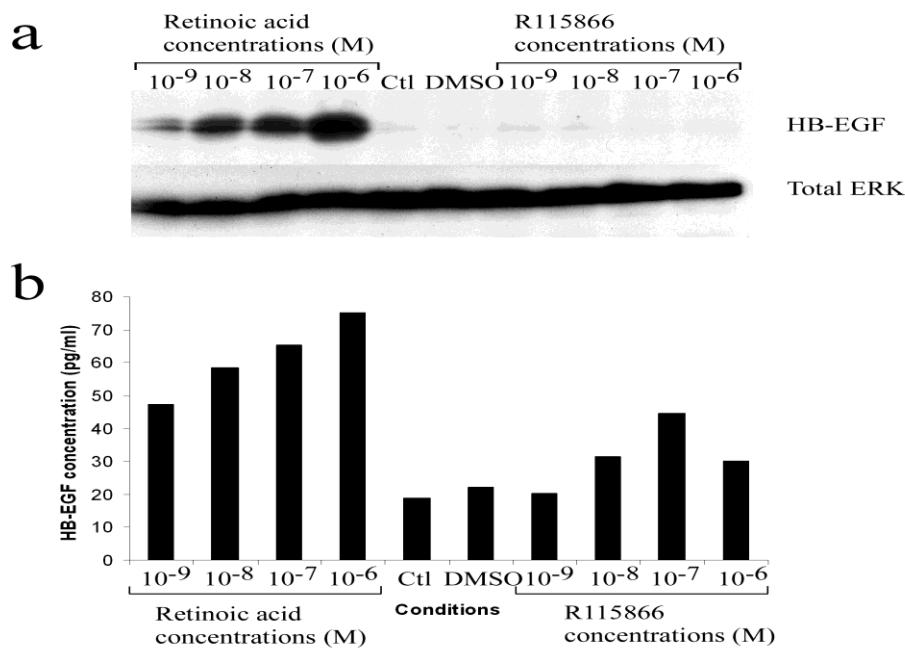

Fig 2: HB-EGF protein expression is induced by RA but not by R115866 treatment.

(a) Confluent keratinocyte cultures were left untreated (Ctl) or treated 18h with increasing RA or R115866 concentrations ranging from 10^{-9} to 10^{-6}M. After treatment, proteins were extracted and analyzed by Western blotting. Detection of the total ERK proteins was used as a loading control. The results shown are representative data obtained in three independent experiments. (b) The culture media were harvested and analyzed by ELISA to measure the concentration of HB-EGF released after 18h of RA treatment or R115866. The results shown represent mean values of two experiments.
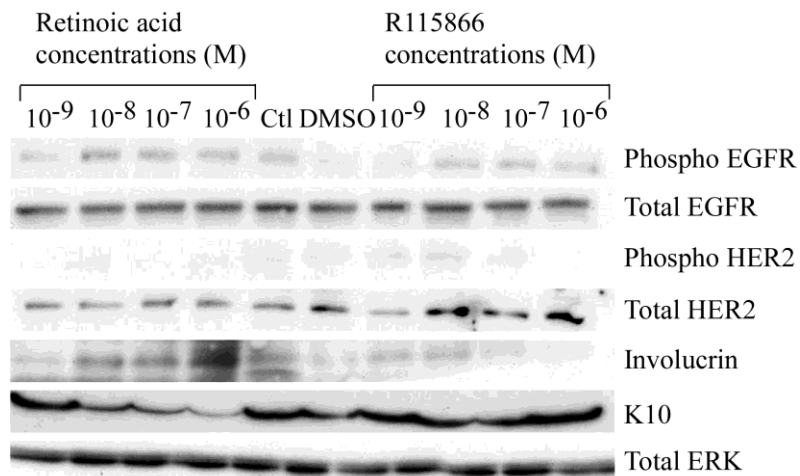
Fig 3: Western blot analysis of keratin 10 and involucrin expression, and analysis of EGFR phosphorylation.

Confluent keratinocyte cultures were left untreated (Ctl) or treated 18h with increasing RA or R115866 concentrations ranging from 10^{-9} to 10^{-6}M. After treatment, proteins were extracted and analyzed by Western blotting. Keratin 10 protein expression is reduced by RA but not by R115866 treatment whereas involucrin protein expression is upregulated. RA or R115866 treatment does not induce the phosphorylation of EGFR or HER2. Detection of the total ERK proteins was used as a loading control.
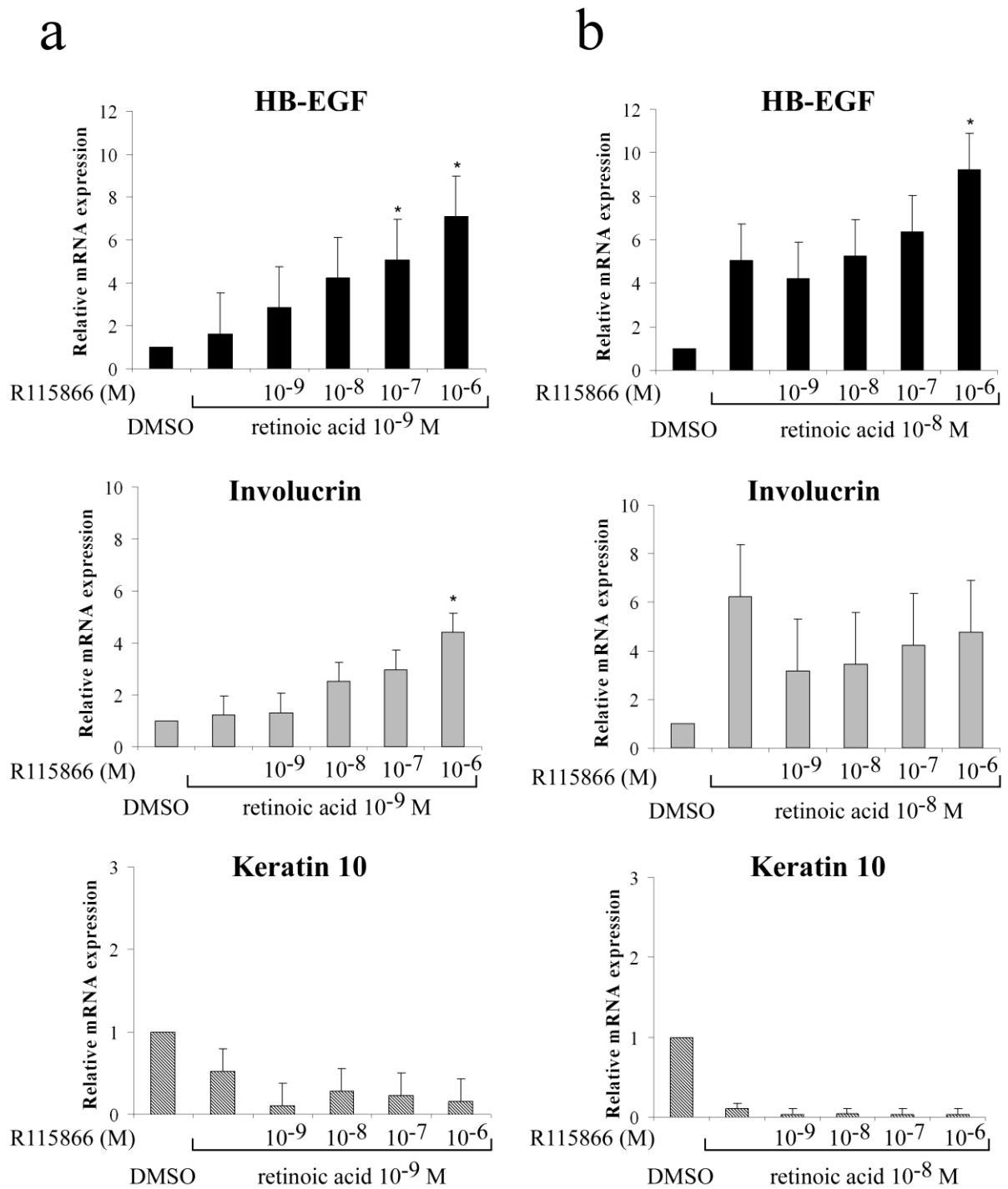
Fig 4: Effect of R115866 in combination with RA (10^{-9} or 10^{-8}M) on HB-EGF, involucrin and keratin 10 gene expression.

Confluent keratinocyte cultures were treated for 18h with R115866 combined with RA at a concentration 10^{-9}M (a) or 10^{-8}M (b). Total RNA extracts were reverse-transcribed into cDNA and analyzed by Real-Time PCR to determine HB-EGF (■), involucrin (▨) or keratin 10 (▨)

gene expression. Data represent three independent experiments and were analyzed by ANOVA I after testing the homogeneity of variance (Bartlett). Post-hoc comparisons were performed by pairwise Scheffe's test. Error bars represent $\pm$ 2 SEM (Standard Error of the Mean) with Student's correction for variance estimation.
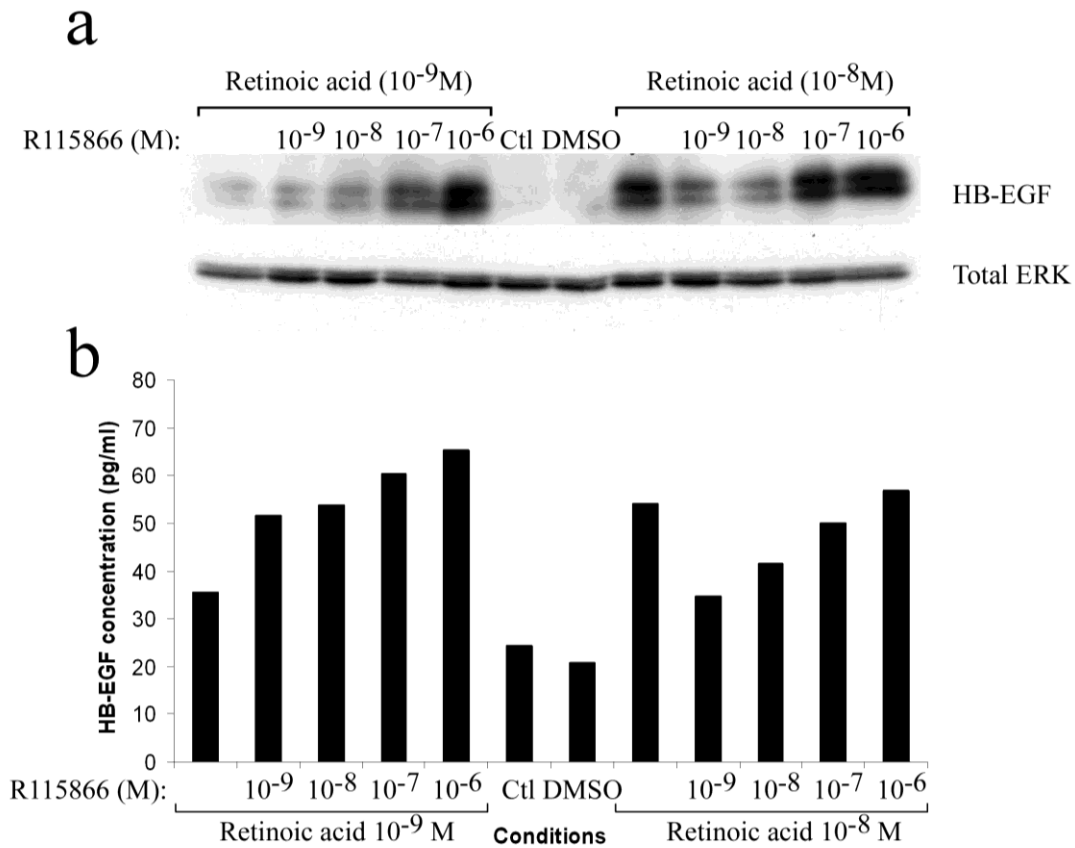

Fig 5: R115866 combined with RA treatment (10^{-9} or 10^{-8}M) increases HB-EGF protein expression.

Confluent keratinocyte cultures were left untreated (Ctl) or treated for 18h with R115866 combined with RA at 10^{-9}M or 10^{-8}M concentration. (a) Proteins were then extracted and analyzed by Western blot. Detection of total ERK protein was used as a loading control. The results shown are representative data obtained in three independent experiments. (b) The culture media were harvested and analyzed by ELISA to measure the concentration of HB-EGF released after 18h of RA treatment or R115866. The results shown represent mean values of two experiments.
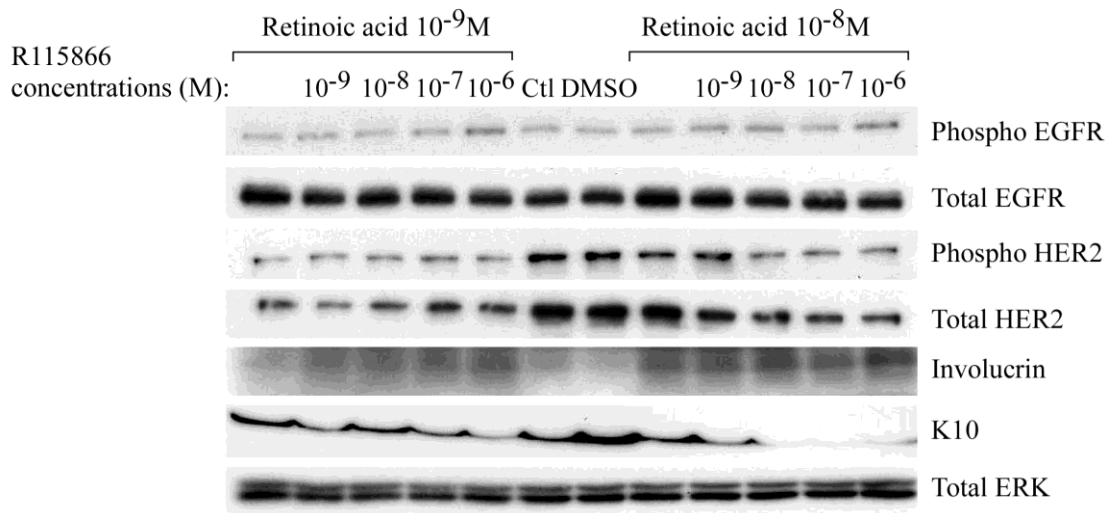
Fig 6: R115866 potentiates the effect of RA on keratin 10 and involucrin expression.

Confluent keratinocyte cultures were left untreated (Ctl) or treated for 18h with R115866 combined with RA at 10^{-9}M or 10^{-8}M concentration. After treatment, proteins were extracted and analyzed by Western blotting. Keratin 10 protein expression is reduced by R115866 combined with RA (10^{-9}M or 10^{-8}M) treatment, whereas involucrin protein expression is upregulated. The combination of R115866 with RA treatment does not modify the phosphorylation of EGFR or HER2. Detection of the total ERK proteins was used as a loading control.
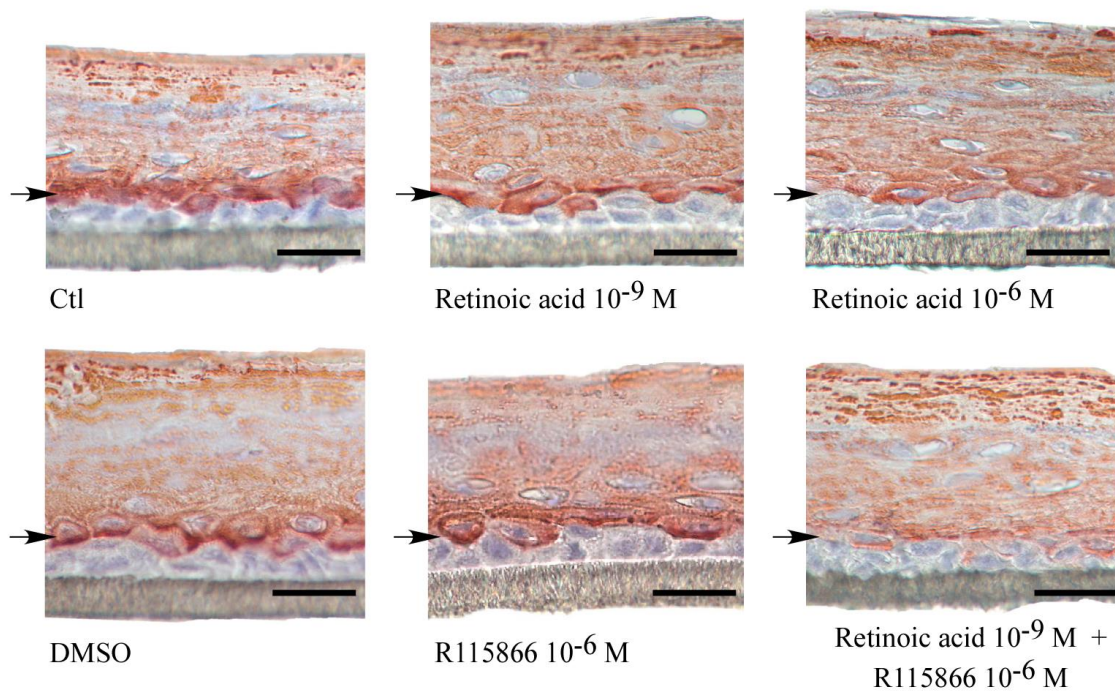
Fig 7: Effect of R115866 and RA on keratin 10 expression in RHE.

At the eleventh days of culture, the RHE were treated during 48h with RA (10^{-9}M or 10^{-6}M) or R115866 (10^{-6}M) or R115866 (10^{-6}M) combined with RA (10^{-9}M). Then, histological sections perpendicular to the surface of RHE were prepared for the immunohistochemical detection of keratin 10. Arrowheads indicate where a decreased labeling of keratin 10 is observed after RA or after R115866 (10^{-6}M) combined with RA (10^{-9}M) treatment. Bars = 25µm.