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*Full Length Research Paper*

# Decreased EGFR mRNA expression in response to antipsoriatic drug dithranol *in vitro*

**Tewin Tencomnao[1*], Chalinee Ronpirin[2], Anchalee Prasansuklab[1] and Yong Poovorawan[3]**

[1]Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand.
[2]Department of Preclinical Science, Faculty of Medicine, Thammasat University, Pathumthani 12121, Thailand.
[3]Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

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**Dithranol is enormously effective in the treatment of psoriasis; however its molecular mode of action should be further elucidated. Since epidermal growth factor receptor (EGFR) is involved in the pathogenesis of psoriasis, the objective of this study was to investigate the transcriptional effect of dithranol on EGFR gene expression in the HaCaT keratinocyte cell line, which is commonly employed as a model system to study psoriasis including experiments examining the effects of therapeutic drugs and cellular regulators on keratinocytes. Cultured HaCaT cells were treated with 0.1-0.5 µg/ml dithranol for 30 min. After 4 h, total cellular RNA isolated from HaCaT cells was reverse transcribed to cDNA which was subjected to polymerase chain reaction (PCR) with specific primer pair for EGFR. We found that dithranol treatment down-regulated the EGFR mRNA of HaCaT cells in a concentration-dependent manner. Our result was further substantiated using a quantitative real-time PCR approach. Taken together, the dithranol-induced down-regulation of the EGFR in cultured human keratinocytes might help to disclose the molecular therapeutic action of the drug.**

**Key words:** Psoriasis, dithranol, EGFR, gene expression, human keratinocytes.

## INTRODUCTION

Psoriasis, a chronic inflammatory skin disease affecting approximately 2-4% of the population worldwide, is not curable although many treatments are available to reduce the bothersome symptoms and appearance of the disease. Dithranol (1, 8-dihydroxy-9-anthrone) also referred to as anthralin is one of the critical topical antipsoriatic agents (Pavithran, 2001). It is a vastly effective compound in the therapy of active psoriasis, but its molecular mechanism of action remains to be appropriately elucidated.

Both transforming growth factor-α (TGF-α) (Gottlieb et al., 1988; Elder et al., 1989) and its receptor, the EGF receptor (EGFR) (Nanney et al., 1986; Choi et al., 1992) have been found to be over-expressed in active psoriatic plaques, thus implicating their functional role in psoriatic hyperplasia. According to the study using normal human keratinocytes isolated from neonatal foreskins, it was demonstrated that pharmacologic concentrations of dithranol decreased TGF-α mRNA expression by Northern blot, and EGFR binding was also inhibited (Gottlieb et al., 1992). In addition, down-regulation of EGFR on human epidermal cell line SCL-II by dithranol was shown using ligand binding assays (Kemény et al., 1993). Recent study showed induced EGFR phosphorylation in keratinocytes by dithranol (Peus et al., 2004), thus highlighting the effect of dithranol on post-translational modification, whereas the influence on EGFR mRNA transcripts was

*Corresponding author. E-mail: tewin.t@chula.ac.th. Phone: (662) 218-1081 ext. 313. Fax: (662) 218-1082.

**Abbreviations: Bp**, Base pairs; **DNase I**, deoxyribonuclease; **FBS**, fetal bovine serum; **DMEM**, dulbecco's modified eagle's medium; **EGF**, epidermal growth factor; **EGFR,** epidermal growth factor receptor; **HB-EGF**, heparin binding EGF-like growth factor; **PBS,** phosphate buffered saline; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **TGF-α**, transforming growth factor-α; **VDRE**, vitamin D-responsive element.

**Table 1.** Specific oligonucleotide primers used in RT-PCR.

| Name | Oligonucleotide sequence (5' → 3') | Product length (bp) | Reference |
|---|---|---|---|
| EGFR-sense | GCTACGATTGGCTGAAGTAC | 518 | Kubota et al. (2001) |
| EGFR-antisense | ATTGGGTGTAGAGAGACTGGA | | |
| β-actin-sense | ACGGGTCACCCACACTGTGC | 656 | Fisker et al. (2004) |
| β-actin-antisense | CTAGAAGCATTTGCGGTGGACGATG | | |

not studied. The first two studies concerning dithranol mentioned here also have certain weak points. Gottlieb et al. (1992) utilized such cellular model that might not be most suitable for psoriasis, and they did not investigate the transcriptional effect of dithranol on EGFR gene expression. Although the inhibition of binding between EGFR and ligands was discovered, suggesting the functional effect of dithranol on altering components of the EGFR pathway in cultured keratinocytes, insights into the EGFR gene expression have not been elucidated. Like the study of Gottlieb et al. (1992), Kemény et al. (1993) revealed their findings based solely on the ligand binding assays, and they employed the squamous cell carcinoma cell line (SCL-II) of human origin, which was also not the frequently analyzed cellular model for psoriasis.

To date, the EGFR and its ligands have been principally recognized to be one of the most powerful and complex signaling networks, especially in diverse effects on skin biology and pathology (Schneider et al., 2008). Herein, the objective of this study was to investigate the transcriptional effect of dithranol on EGFR gene expression in the HaCaT keratinocyte cell line. HaCaT cells were established as the first permanent epithelial cell line from adult human skin, and has been evident to exhibit normal differentiation, thus providing a promising tool for studying regulation of keratinization in human cells (Boukamp et al., 1988). The HaCaT cell line has been the most widely used cellular model to study hyperproliferative skin diseases such as psoriasis (Ryle et al., 1989; Ockenfels et al., 1995; Lehmann, 1997; Paramio et al., 1998, 1999; Garach-Jehoshua et al., 1999; Farkas et al., 2001, 2003; Kim et al., 2007; Belsõ et al., 2008). Thus, HaCaT keratinocytes were chosen as proper candidates for studying the molecular effect of such therapeutic drug as dithranol on the EGFR gene expression.

## MATERIALS AND METHODS

### Cell culture

The human keratinocyte cell line HaCaT (kindly provided by Dr. N.E. Fusenig, German Cancer Research Centre, Heidelberg, Germany) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and cells were maintained at 37°C in a humidified atmosphere at 5% $CO_2$.

### Dithranol treatment

Dithranol treatment was performed following a procedure previously described by Farkas et al. (2001) with minor modifications. Briefly, cultured HaCaT cells at about 90% confluence in a 60 mm plate were washed with phosphate buffered saline (PBS) under sterile conditions and were incubated for 30 min at 37°C in a humidified atmosphere at 5% $CO_2$ with increasing concentrations of dithranol (0.1, 0.25 and 0.5 μg/ml) (Sigma, St. Louis, MO) in DMEM containing 0.5% FBS. Dithranol was always freshly dissolved in acetone and used immediately. Control cells were treated with acetone only or left untreated.

### Total RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

After dithranol treatment, treated cells were washed with PBS and were incubated in DMEM containing 10% FBS for 4 h at 37°C in a humidified atmosphere at 5% $CO_2$ prior to total cellular RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amount of RNA was determined by absorbance at 260 nm. Prior to RT-PCR reaction, about 1 μg of the total RNA was treated with $10^{-5}$ U deoxyribonuclease I (DNase I) (Invitrogen, Carlsbad, CA) for 15 min at 25°C. First-strand cDNA was synthesized from about 100 ng of DNase I-treated RNA using ImProm-II™ Reverse Transcription System (Promega, Madison, WI) with oligo$(dT)_{17}$ primer following the manufacturer's protocol. Reverse transcription was carried out at 50°C for 60 min. After cDNA synthesis, the desired DNA fragment was then amplified for 30-35 cycles using RealTaq DNA Polymerase (Real Biotech Corp., Taipei, Taiwan) and specific primers listed in Table 1 for EGFR transcripts and β-actin transcripts as controls. Each cycle of PCR consisted of a 30 s denaturation at 94°C, a 30 s annealing at 52°C for EGFR and 58°C for β-actin, and a 45 s extension at 72°C. The products of RT-PCR were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The DNA bands corresponding to EGFR and β-actin transcripts were 518 base pairs (bp) and 656 bp, respectively.

### Quantitative real-time PCR

Real-time PCR was performed on a LightCycler instrument using the LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I Kit according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Together with the specific primers, the master mix was added to cDNA solutions, including water blanks as negative controls. The PCR was run for 10 min at 95°C and followed by 50 cycles of 10 s at 95°C, 10 s at 52°C and 35 s at 72°C. After amplification, melting curve analysis was performed to verify specificity. For data analysis, we used the LightCycler Data Analysis software 4.0. The expression of EGFR target gene was normalized to that of β-actin, and the data for each dithranol concentration treatment represented fold change in normalized

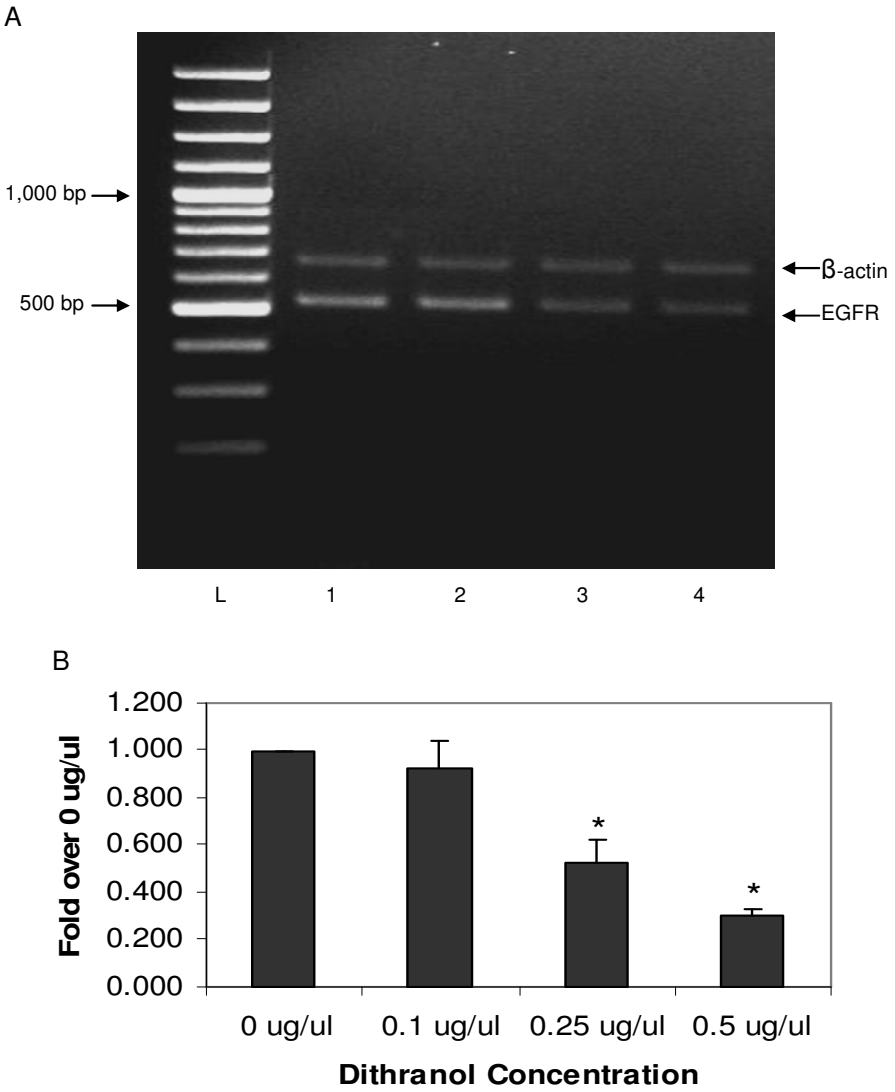


**Figure 1.** RT-PCR analysis of EGFR expression in HaCaT cells treated with increasing dithranol concentrations (0, 0.1, 0.25 and 0.5 ug/ml) for 4 h. (A) 2% agarose gel electrophoresis showing mRNA transcripts of EGFR and β-actin. Lane L: 100-bp DNA ladders, whereas lanes 1 to 4 corresponding to increasing dithranol concentrations used for treating HaCaT cells. (B) Average fold over expression at 0 ug/ml dithranol. A symbol, *, indicating a statistically significant difference ($P < 0.05$).

mRNA expression relative to that at 0 ug/ml of dithranol. The relative expression in fold change was arbitrarily set at 1. To compare expression results between each dithranol concentration treated, a relative quantification model was used (Pfaffl, 2001).

### Statistical analysis

All band densities was analyzed by 1D-Multi Lane Densitometry program in an AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA). The EGFR density from each dithranol concentration was normalized to β-actin density. Each experiment was performed in triplicate, and data were reported as fold over 0 μg/ml dithranol concentration. The data represented the mean ± S.D. of three independent experiments and were analyzed by the Student's *t*-test. Differences at $P < 0.05$ were considered to be significant.

## RESULT AND DISCUSSION

To study the effect of dithranol on EGFR mRNA expression, we performed the semi-quantitative RT-PCR approach. After 4 h of treatment, our result showed that normalized EGFR mRNA transcripts of HaCaT cells were decreased as dithranol concentrations used were increasing (Figure 1). As compared to 0 ug/ml dithranol concentration, the difference in EGFR mRNA transcripts was statistically significant ($P < 0.05$) when 0.25 and 0.5 ug/ml

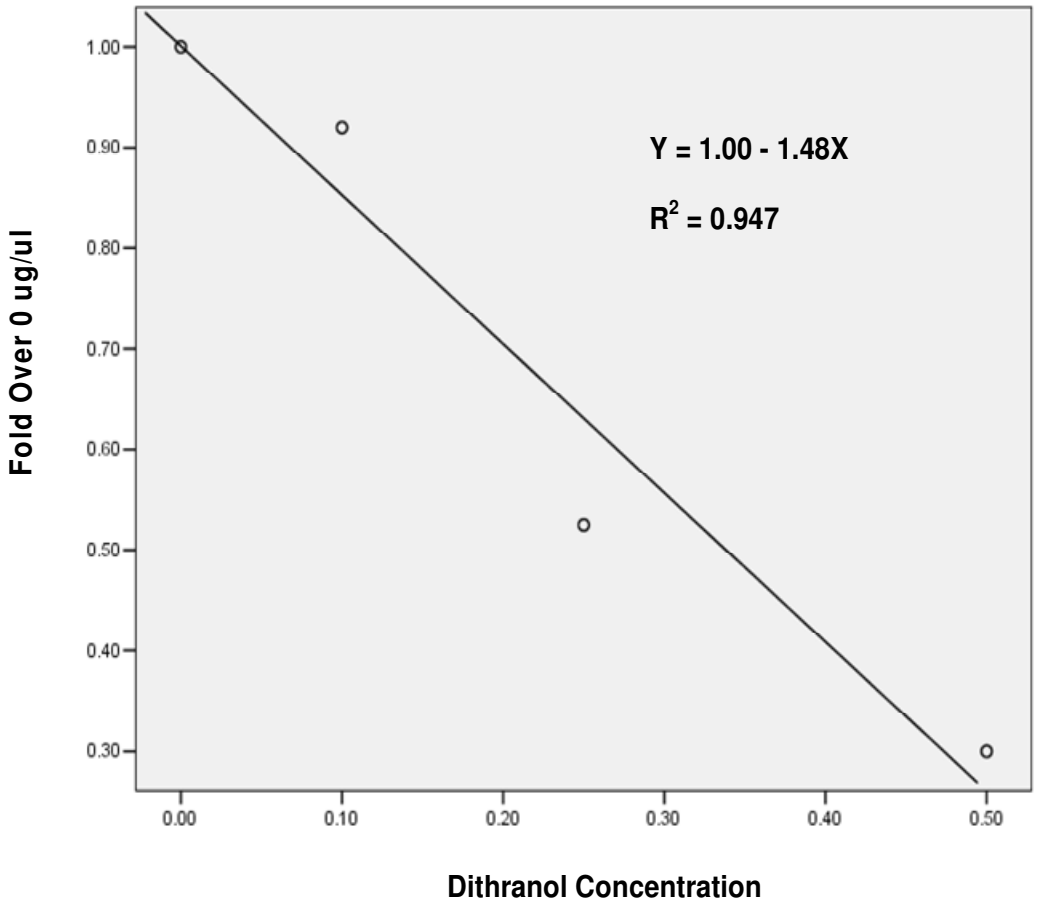


**Figure 2.** Pearson's correlation between normalized EGF receptor expression levels in HaCaT cells and concentrations of dithranol for treatment.

dithranol were used for treatment. The down-regulation effect of dithranol was concentration-dependent as analyzed using Pearson's correlation (Figure 2). In addition, the quantitative measurement of EGFR transcripts was carried out using the real-time PCR method. We found that normalized EGFR mRNA transcripts, as dithranol concentrations increasing from 0.1, 0.25 and 0.5 ug/ml were down-regulated to 0.60, 0.70 and 0.48-fold over that of 0 ug/ml dithranol treatment, respectively.

Therefore, dithranol down-regulated the EGFR mRNA expression of HaCaT cells in a dose-dependent manner. However, inconsistent result was found between the two experimental approaches. The semi-quantitative RT-PCR technique showed significant reduction of normalized EGFR mRNA transcripts when HaCaT cells were treated with dithranol concentrations of 0.25 and 0.5 ug/ml, whereas we could observe that significant drop using the quantitative real-time PCR approach even in the presence of dithranol concentration as low as 0.1 ug/ml for treatment. This might be explained by the difference in sensitivity between the two techniques. Using the quantitative real-time PCR, normalized EGFR mRNA transcripts were found to be 0.60 and 0.70-fold over that of 0 ug/ml dithranol in response to treatment with dithranol concentrations of 0.1 and 0.25 ug/ml, respectively. However, these levels of normalized EGFR mRNA were comparable. In the present study, we utilized only one housekeeping gene, β-actin, for normalization since it has been often used for this regard. It should be noted using more than one reference genes is better for assuring data reliability.

Previous studies have shown that various cytokines and growth factors are over-expressed in psoriatic epidermis (Schon and Boehncke, 2005). Keratinocytes are the major component cells of the epidermis, and their growth is controlled by both negative and positive mediators (Hashimoto, 2000). Of these mediators, the most crucial mechanism for the proliferation of keratinocytes is the signal from the EGFR. The EGF family consists of EGF, TGF-α, heparin binding EGF-like growth factor (HB-EGF), amphiregulin, epiregulin, betacellulin, epigen, neuregulin (NRG)-1, NRG-2, NRG-3, and NRG-4, and the EGFR family consists of EGFR (also called ErbB1, ErbB2, ErbB3, and ErbB4 (Hashimoto, 2000). Previous reports have shown that TGF-α, amphiregulin and HB-EGF are over-expressed in psoriatic epidermis (Gottlieb

et al., 1988; Elder et al., 1989; Cook et al., 1992; Stoll and Elder, 1998). Recent evidence also demonstrates an increase in the expression of epiregulin in psoriatic epidermis (Shirakata et al., 2007).

The EGFR plays a vital part in growth, differentiation, and motility of normal as well as cells in hyperproliferative states. For predictive disease diagnostics and therapeutic targeting of EGFR, it is critical to know how the expression level of EGFR is controlled and related to receptor signaling. Mechanisms of EGFR gene transcription modulation depend primarily on regulatory regions including a gene promoter, three enhancers and polymorphisms (e.g. a CA repeat in intron 1) (Brandt et al., 2006). For instance, there is recent evidence that this CA repeat polymorphism of EGFR gene is associated with the occurrence of skin rash with gefitinib treatment, a drug used in the treatment of certain types of cancer (Huang et al., 2008).

In addition to dithranol, such drugs as calcitriol and calcipotriol are widely used in the tropical treatment of psoriasis (Körver et al., 2007). Since a candidate vitamin D-responsive element (VDRE) is known to locate in the EGFR gene promoter, there is a study demonstrating that EGFR mRNA transcripts in UMR 106-01 osteoblast-like cells are altered in the presence of calcitriol treatment, but this effect occurs due to calcitriol acting on EGFR mRNA stability, not gene transcription (González et al., 2002). In our case, we do not know whether dithranol acts on gene transcription or mRNA stability to modulate the EGFR mRNA levels in HaCaT keratinocytes.

Farkas et al. (2001) could observe the changes in mRNA levels of IL-10 receptors in HaCaT cells even when cells were treated with dithranol for 2 h. In the present investigation, we treated HaCaT cells for 4 h to assure the cellular signaling alterations. Regarding psoriasis, understanding its pathogenesis and therapy in a more advanced level is sill needed, thus encouraging more research outcomes in the field (Krueger and Ellis, 2005). The same is true for HaCaT research since recent evidence establishes *in vitro* culture condition to study HaCaT cells (Deyrieux and Wilson, 2007). In conclusion, we found that dithranol treatment down-regulated the EGFR mRNA of HaCaT cells in a concentration-dependent manner, thus providing an insight into the molecular therapeutic action of this drug.

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