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# Therapeutic and Cytotoxic Effects of the Novel Antipsoriasis Codrug, Naproxyl−Dithranol, on HaCaT Cells

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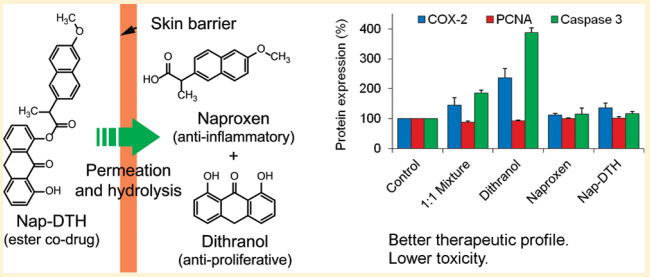
**ABSTRACT:** A novel topical codrug, naproxyl−dithranol (Nap-DTH), in which dithranol and naproxen are linked via an ester in a 1:1 ratio to form a single chemical entity, was synthesized. The antiproliferative, anti-inflammatory and toxic effects of Nap-DTH were assessed, at the cellular level, using various *in vitro* methods. Cultured HaCaT keratinocytes were treated with Nap-DTH, and the cellular effects were compared with those of the parent compounds, individually and as a 1:1 mixture of naproxen:dithranol to mimic 1:1 *in situ* liberation from Nap-DTH. The results demonstrate that Nap-DTH did not modify proliferation and only exhibited slight toxic effects after 24 h at concentrations >21 $\mu$M. At a lower concentration (3.4 $\mu$M), Nap-DTH did not alter cell proliferation or inflammation, which suggests that the codrug is therapeutically inert. Relating to this, the 1:1 mixture of naproxen:dithranol exhibited the lowest toxic effect and the highest antiproliferative effect on HaCaT keratinocytes compared to dithranol at the same concentration. Moreover, the 1:1 mixture exhibited a reduced inflammatory effect compared to dithranol alone, as reflected by the upregulation of cyclooxygenase-2 by 45% and 136%, respectively. In spite of the 1:1 mixture showing a greater downregulation of Ki-67 and a 2-fold reduction of proliferating cell nuclear antigen (both cellular markers of proliferation) than dithranol, dithranol showed a much greater induction of cleaved caspase-3 protein expression (upregulated by 287%, compared to 85% for the 1:1 mixture). This suggests that when dithranol was administered with naproxen, inhibition of cell growth plays a more important role in the antiproliferation effects than the induction of apoptotic cell death. These results confirm that the codrug would lead to a better therapeutic profile and fewer adverse effects compared to its parent compounds.




**KEYWORDS:** codrug, prodrug, naproxen, dithranol, inflammation, proliferation

## INTRODUCTION

Psoriasis is one of the most common skin diseases, affecting approximately 2% of the general population.[1] It is a chronic, autoimmune, inflammatory, proliferative and scaling skin disorder. The pathophysiology of psoriasis is complex and the exact etiology is yet unknown. Psoriasis can be disabling and has important psychological, social and economic consequences. In some instances, the condition may be life-threatening.[2] Epidemiological studies have shown that approximately a third of psoriatic patients develop psoriatic arthritis, an inflammatory condition associated with joint pain and swelling, thus necessitating treatment of both skin and joints.[3,4]

Despite the vast number of antipsoriatic and antiarthritic treatments currently available, their adverse effects and inadequate efficacy have created the need for better-tolerated and more effective therapies. Combination therapy is common in the management of psoriasis; it is often more efficacious and better tolerated than monotherapy.[5] For this reason, a codrug approach would be of great advantage in the treatment of psoriasis. A codrug is a chemical entity comprising two or more therapeutic compounds, each active against the same disease, linked by a cleavable covalent bond. The advantages of codrugs over coadministration or coformulation in topical drug delivery have been reviewed previously.[6]

A novel antipsoriatic codrug, naproxyl−dithranol (Nap-DTH) consisting of dithranol and naproxen, has been previously synthesized by our group[7] (Figure 1). Its degradation, stability, skin penetration and permeation, as well as skin staining effects, have been reported.[7,8] Our data suggested that Nap-DTH exhibited minimal skin staining effects, which is a major compliance challenge for this highly effective antipsoriatic drug, compared to dithranol.[8] Furthermore, greater skin retention of Nap-DTH was observed compared with the parent compounds. These results suggest that not only may Nap-DTH enhance the topical delivery of naproxen and dithranol in the codrug format but also it may reduce the autodegradation of dithranol as well as minimize systemic side effects by limiting the systemic bioavailability of naproxen.

Since both hyperproliferative and inflammatory processes are implicated in psoriasis, an effective antipsoriatic compound should target both of these disease components. The current work evaluated the potential *in vitro* antiproliferative and anti-inflammatory activities, as well as any potential toxic effects, of

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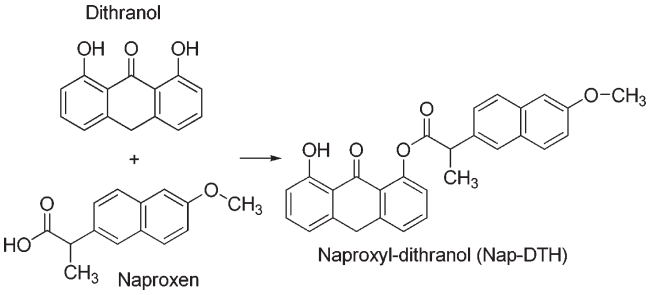


**Figure 1.** Formation of naproxyl−dithranol codrug in a 1:1 ratio of dithranol and naproxen.

Nap-DTH, as compared to naproxen and dithranol individually. The immortalized, nontumorigenic human keratinocyte cell line, HaCaT,[9] has been widely utilized to evaluate antiproliferative agents.[10,11] The cell line has also been shown to be sensitive to the antiproliferative action of dithranol.[12] Thus, the HaCaT cell line was used as the *in vitro* keratinocyte model in the current work.

Ki-67 is a cellular marker of proliferation and can be used to discern actively proliferating cells. The monoclonal antibody, MIB-1, developed against the Ki-67 antigen, is commonly used for the detection of Ki-67.[13] The expression of Ki-67 has been shown to be significantly higher in the psoriatic epidermis compared to normal human epidermis.[14,15] Thus, quantification of Ki-67 expression could provide a good indication as to how Nap-DTH affects keratinocyte proliferation. COX-2 is induced in inflammatory diseases such as psoriasis[16,17] and is the therapeutic target of many anti-inflammatory drugs, such as naproxen.[18] COX-2 is also reportedly associated with cell differentiation and transformation of keratinocytes.[19] In addition, dithranol is known to induce inflammation which is thought to be due to the generation of free radicals. Thus, COX-2 expression was examined in human HaCaT keratinocytes to compare the effect of Nap-DTH on COX-2 expression with that of the parent compounds.

## ■ MATERIALS AND METHODS

**Materials.** Dithranol was purchased from BUFA Pharmaceutical Products (Uitgeest, Holland). The HaCaT cell line was provided by Stiefel Laboratories, U.K. Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were purchased from Invitrogen Ltd. (Paisley, U.K.). Naproxen, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and fluorescein diacetate (FDA) were obtained from Sigma-Aldrich Company Ltd. (Poole, U.K.). CytoTox-ONE Homogeneous Membrane Integrity Assay kit was purchased from Promega (Southampton, U.K.). The synthesis of Nap-DTH has been described elsewhere.[7] Cyclooxygenase-2 (COX-2) and cleaved caspase-3 antibody were purchased from Cell Signaling Technology (Boston, MA). MIB-1 and EnVision+ System were purchased from Dako (Ely, U.K.). Proliferative cell nuclear antigen (PCNA) was from Insight Biotechnology, U.K. Western blot reagents were from Roche Diagnostics GmbH (Mannheim, Germany). Chemiluminescent SuperSignal West HRP substrate was from Pierce and Warriner Ltd. (Chester, U.K.). All other reagents were obtained from Fisher Scientific U.K. (Loughborough, U.K.).

**Cell Culture.** HaCaT cells were cultured in DMEM, supplemented with 10% FBS (hereafter referred to as "culture medium"), in humidified air with 5% $CO_2$ at 37 °C.

**MTT Assay.** HaCaT cells were seeded into 96-well plates at a cell density of $1.5 \times 10^4$ cells per well. After overnight incubation, the cells were exposed to drug treatment for 5 days. Naproxen, dithranol, Nap-DTH and 1:1 molar ratio of naproxen:dithranol solutions with a range of drug concentrations (5 mM to 0.01 $\mu$M) were prepared in 0.5% DMSO with culture medium. The change of drug solution took place at day 2. Each drug concentration and the vehicle-only control had six replicates in the same plate, with 6 other wells containing culture medium only to verify normal cell growth. After the treatment period, 20 $\mu$L of MTT solution was added to each well and the cells were incubated for 4 h before spectrophotometric analysis at 550 nm, using a microtiter plate reader (Sunrise, Tecan Trading, Switzerland). All assays were performed in triplicate.

**Lactate Dehydrogenase Assay.** HaCaT cells were seeded into a 96-well plate at a density of $2 \times 10^4$ cells per well. After overnight incubation, the cells were exposed to drug solutions (prepared in 0.5% DMSO with culture medium, to a range of IC values derived from the MTT assay to allow direct comparison). Three different controls were used: (1) HaCaT-free control, in which the well contained the vehicle only, to determine background fluorescence; (2) untreated cell control, in which the well contained HaCaT cells with vehicle only, and (3) a 100% lactate dehydrogenase (LDH) release control where the cells were fully lysed prior to the LDH assay, to determine the maximum amount of LDH present. The LDH assay was performed after the treatment period (24, 48, or 72 h), using the CytoTox-ONE kit according to the manufacturer's protocol. Fluorescence was measured using a FLUOstar OPTIMA fluorometer (BMG Labtechnologies, Germany) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Data are represented as mean values of three replicates for each concentration derived from three independent experiments ($n$ = 3), normalized against their respective vehicle controls. Cytotoxicity was expressed as the ratio of the amount of LDH released, per treatment, to the maximum amount of LDH released from the nontreated control cells.

**Flow Cytometry.** HaCaT cells were seeded into a 24-well plate at a density of $5 \times 10^4$ cells per well for overnight before drug treatment as described earlier. After the treatment period (24, 48, or 72 h), all cells were collected and resuspended in DPBS containing 5 $\mu$g mL$^{-1}$ propidium iodide (PI) and 10 $\mu$g mL$^{-1}$ fluorescein diacetate (FDA). A sample of 20,000 cells for each treatment was aspirated into the flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) for analysis using the CELLQUEST software. The experiment was carried out in 3 independent experiments ($n$ = 3). The percentage of PI and FDA stained cells was normalized against the vehicle control.

**Immunocytochemistry.** HaCaT cells were seeded onto a 3-triethoxysilylpropylamine (TESPA)-coated coverslip (coated in-house). The cells were treated with drugs the next day for 3, 6, 9, and 24 h at their respective $IC_{50}$ and at an equimolar concentration (3.4 $\mu$M). The controls contained the vehicle only. Thereafter, the cells were fixed in 10% formalin. The abundance of Ki-67 (a marker of cellular proliferation) and cyclooxygenase (COX)-2 (a marker of inflammation) was

evaluated using EnVision System with MIB-1 antibody and anti-COX-2 antibody, respectively, in three independent experiments according to their manufacturers' protocols. The slides were examined visually and images captured using an Olympus BH-2 microscope fitted with an Olympus DP-12 digital camera system (Olympus, Oxford, U.K.).

**Western Blot Analysis.** HaCaT cells were treated with drugs at $IC_{50}$ and 3.4 $\mu$M for 9 or 48 h. The expression of Ki-67, COX-2, PCNA and cleaved caspase-3 in HaCaT cells was determined using Western blot analysis. In brief, cells were lysed and the cell lysates were centrifugated (IEC Micromax RF microcentrifuge, Thermo Electron Corporation, U.K.) at 300 RCF at 4 °C for 15 min. The proteins (40 $\mu$g) were separated using a 10% sodium dodecyl sulfate polyacrylamide (SDS−PAGE) gel. The proteins were transferred onto a nitrocellulose membrane, and the proteins were immunoblotted with primary antibodies (MIB-1 (1:1000), anti-COX-2 (1:1000), anti-PCNA (1:8000) and anticleaved caspase-3 (1:1000)). The appropriate secondary antibody was used (1:1000 anti-mouse or anti-rabbit IgG antibody). Nitrocellulose papers were developed using an enhanced chemiluminescent system (Thermo Fisher Scientific Inc., USA). $\beta$-Actin was analyzed in parallel to serve as loading control. All experiments were repeated three times ($n$ = 3).

**Statistical Analysis.** Statistical analysis and determination of IC values were undertaken using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, California). Data were compared using a one way analysis of variance (ANOVA) with Tukey's post test or Dunnett's post test. Results are expressed as means ± SD unless otherwise stated. Statistical significance was considered where $p < 0.05$.

**Table 1. The Range of IC Values ($\mu$M; Mean ± SD) for Nap-DTH, Naproxen, Dithranol and a 1:1 Mixture of Naproxen and Dithranol, Derived from the MTT Assay Following 5-Day Treatment of HaCaT cells[a]**

| compd | Nap-DTH | naproxen | dithranol | 1:1 mixture |
|---|---|---|---|---|
| $IC_{70}$ | 20.8 ± 1.6 | 1200 ± 120 | 1.6 ± 0.07 | 1.5 ± 0.04 |
| $IC_{60}$ | 33.7 ± 2.6 | 1900 ± 180 | 2.6 ± 0.11 | 2.5 ± 0.07 |
| $IC_{50}$ | 52.7 ± 4.1 | 2500 ± 240 | 3.6 ± 0.16 | 3.4 ± 0.10 |
| $IC_{40}$ | 83.6 ± 6.5 | 3400 ± 320 | 5.2 ± 0.23 | 4.5 ± 0.13 |
| $IC_{30}$ | 99.0 ± 6.9 | 3900 ± 370 | 6.4 ± 0.28 | 6.1 ± 0.17 |

[a] Data are from 3 independent experiments ($n$ = 3).

## RESULTS

**Cell Proliferation and Cytotoxicity.** The effect of Nap-DTH and the parent compounds (naproxen and dithranol) on cell proliferation was determined using the MTT assay. A range of IC values were calculated (Table 1). The order of potency was determined as 1:1 mixture of naproxen:dithranol ≈ dithranol > Nap-DTH > naproxen.

The amounts of LDH released from HaCaT cells following drug treatment are shown in Figure 2. At all IC values tested, LDH release was significantly greater ($p < 0.05$) following treatment with Nap-DTH or naproxen, compared to dithranol or the 1:1 mixture. In the first 24 h, the greatest LDH release was seen with Nap-DTH treatment. However, the profile of LDH release changed gradually over the course of 72 h (Figure 2A), i.e. LDH release from Nap-DTH-treated cells gradually decreased, but continued to rise with other treatments, such that by 72 h,

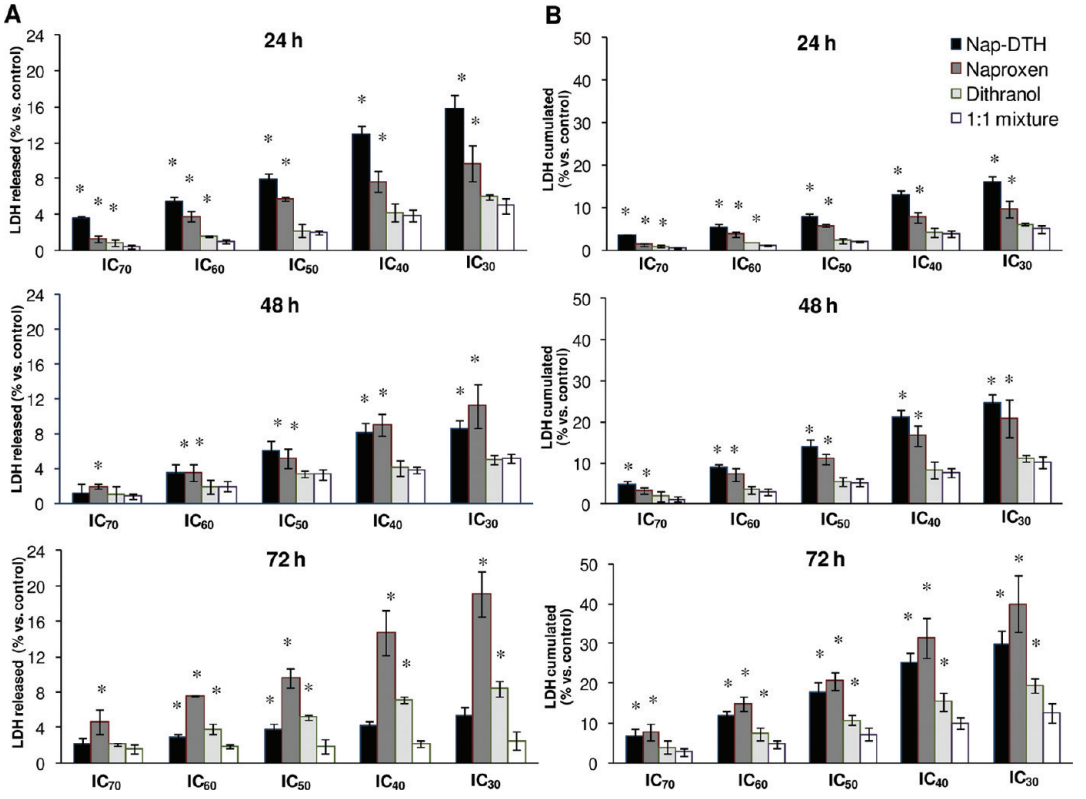


**Figure 2.** LDH released from HaCaT cells after 24 h, 48 h and 72 h of incubation with Nap-DTH, naproxen, dithranol and 1:1 mixture of naproxen: dithranol: (A) amount released and (B) accumulated amount (mean ± SD, % normalized against the control, $n$ = 3, *$p < 0.05$ compared to 1:1 mixture).


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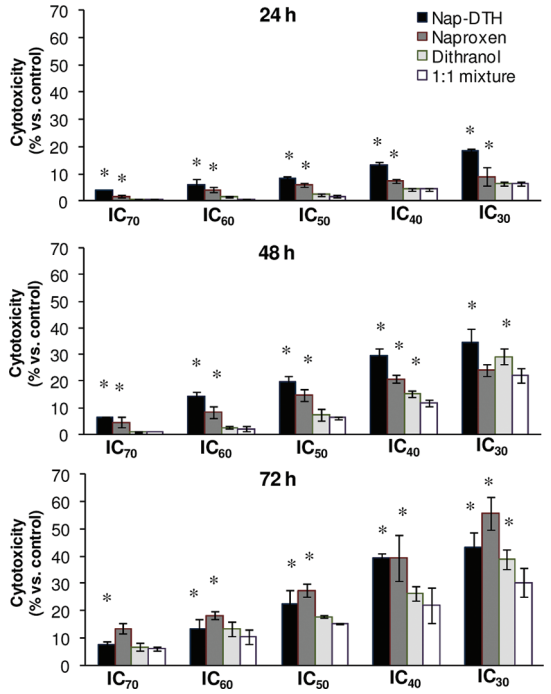


**Figure 3.** Cytotoxicity determined by duo-staining of HaCaT cells with PI and FDA following 24 h, 48 and 72 h incubation with Nap-DTH, naproxen, dithranol and 1:1 mixture of naproxen:dithranol and analyzed by flow cytometry (mean ± SD, normalized against vehicle control, $n$ = 3 and shown as percentage of nonviable cells in the total cell population; $^*p < 0.05$ compared to 1:1 mixture).

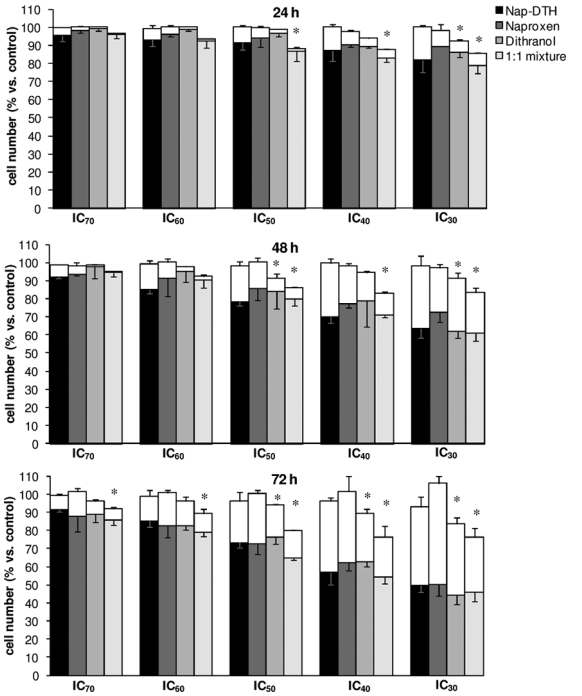


**Figure 4.** Cell proliferation after 24, 48, and 72 h incubation of the HaCaT with Nap-DTH, naproxen, dithranol and 1:1 mixture of naproxen:dithranol. Top section of the bar indicates the % of nonviable cells from flow cytometric analysis, bottom bar is % of viable cells from the MTT assay and the combined bar gives an indication of cell growth inhibition (mean ± SD, $n$ = 3 and expressed as % of cell population; $^*p < 0.05$ indicates a significant difference compared with the total cell population of the control).

LDH release from naproxen-treated cells had surpassed that from Nap-DTH treatment at all concentrations tested. The data show that Nap-DTH at concentrations >34 $\mu$M ($IC_{60}$) rapidly caused significant cell damage within the first 24 h. Conversely, naproxen showed similar levels of LDH release throughout the first 48 h and a significantly higher level of LDH release at 72 h ($p < 0.05$).

For dithranol and the 1:1 drug mixture, which mimics the 1:1 liberation of Nap-DTH, both treatments showed no significant evidence of cell death compared to the vehicle control ($p > 0.05$) at 24 h until their $IC_{30}$ (6.4 and 6.1 $\mu$M, respectively) were used. At 72 h, dithranol caused a significantly higher percentage of cell death compared to the 1:1 mixture ($p < 0.05$). The 1:1 mixture demonstrated the lowest cell damage. The 1:1 mixture $IC_{50}$ value was very similar to that of dithranol but has a significantly lower amount of LDH released at 72 h. The cumulative concentration of LDH is also calculated (Figure 2B). Over the course of 72 h, naproxen treatment gave the highest cumulative concentration of LDH release, while the 1:1 mixture gave the lowest.

Flow cytometry results are presented as cytotoxicity to allow direct comparison with the LDH results. The data are shown as the percentage of nonviable cells (PI-stained cells) in the total cell population (total PI- and FDA-stained cells minus debris), normalized against the vehicle control (Figure 3). Cell viability thus determined corroborates the results of the LDH assay. At 24 h, cytotoxicity was greatest with the use of Nap-DTH at all IC tested, followed by naproxen. The difference in cytotoxicity between dithranol and the 1:1 mixture was not significant ($p < 0.05$).

To ascertain that the antiproliferative effect seen in the MTT assay was due to growth inhibition and not cell death, viable cell data from the MTT assay and cell death data from the flow cytometric analysis were collated and plotted on the same axes (Figure 4). This gave the combined proportion of both viable and nonviable cells relative to the vehicle control. Since the total cell number in the vehicle control represented 100% of cells (viable or otherwise) under proliferative conditions, any shortfall observed in the combined data was attributed to nonproliferation rather than cell death.

With Nap-DTH, the cells showed no significant reduction in proliferation ($p > 0.05$) even after 3 days of incubation. The reduction in cell proliferation seen in the MTT assay was perhaps attributable to nonspecific cytotoxic effects of Nap-DTH. Some reductions in cell proliferation are seen with Nap-DTH, particularly at 72 h and $IC_{30}$ (Figure 4), although these are not statistically significant ($p = 0.26$) compared to the vehicle control. It is conceivable that such reductions could be the result of hydrolysis of the codrug to liberate the parent compounds within the cells. Indeed, in separate analyses, Nap-DTH has been shown to liberate the parent moieties in intact porcine ear skin,[8] as well as in the presence of porcine skin homogenate or porcine liver esterases.[7] Treatment with naproxen did not lead to significant cell growth inhibition. However, dithranol demonstrated significant growth inhibition from 24 h onward following incubation at $IC_{30}$ or greater. The 1:1 mixture of naproxen:dithranol had the greatest antiproliferation effect of all treatments, at all concentrations and at all incubation times.

**Immunocytochemical Analysis.** Immunocytochemistry (ICC) was performed to assess levels of the proliferation marker, Ki-67, and the inflammatory marker, COX-2. HaCaT cells, being highly

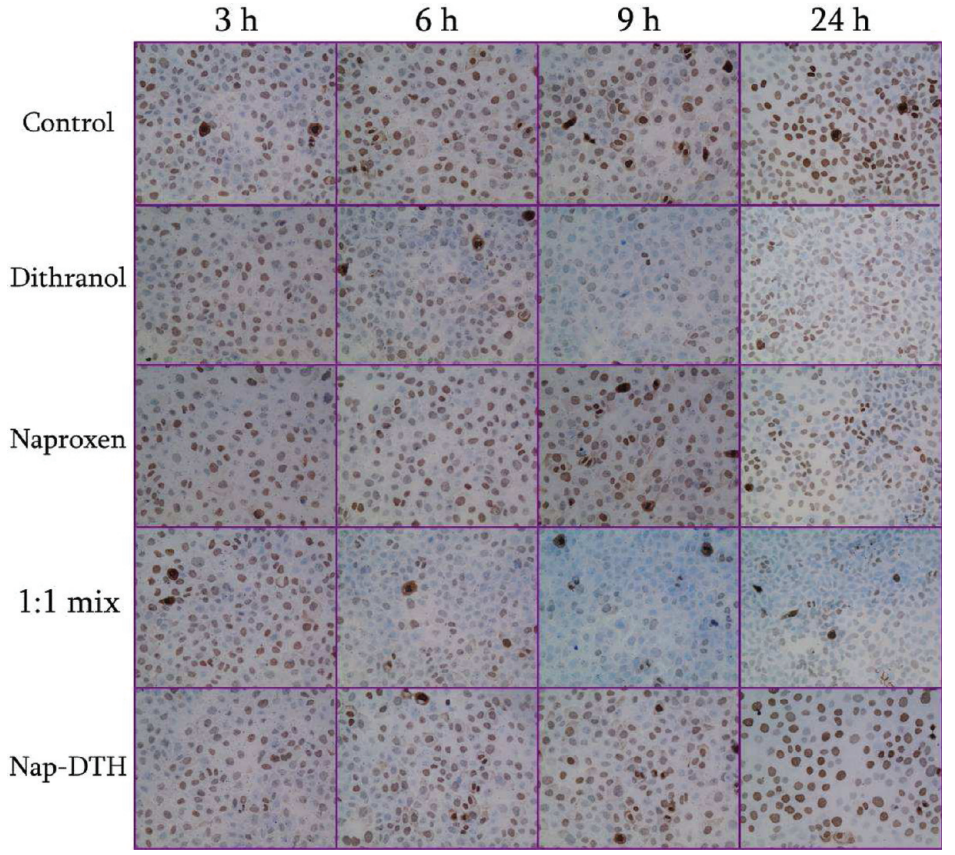


**Figure 5.** Representative images of ICC assay to detect Ki-67 in HaCaT keratinocytes after incubation with different drug treatments at their respective $IC_{50}$ for 3, 6, 9, and 24 h: dithranol ($IC_{50}$ = 3.6 $\mu$M); naproxen ($IC_{50}$ = 2.5 mM); 1:1 mixture of naproxen:dithranol ($IC_{50}$ = 3.4 $\mu$M) and Nap-DTH ($IC_{50}$ = 52.7 $\mu$M). The control contained the vehicle (0.5% DMSO, 10% FBS in DMEM) only (original magnification ×200).

proliferative keratinocytes, express high levels of Ki-67. This is evident from the intense nuclear staining of the control cells, without drug treatment, at all time points (Figures 5 and 7). This allows any knockdown effect to be easily observed and compared between the treatment groups, both at $IC_{50}$ (Figure 5) and at the equimolar concentration of 3.4 $\mu$M (Figure 7).

The expression of COX-2 is induced by a range of stimuli, including tissue damage and inflammation. Unchallenged HaCaT cells show low levels of COX-2 expression,[10,20] thus any upregulation of COX-2 due to induction of inflammation by the drug treatments would be easily observed. COX-2 expression levels after drug treatment at $IC_{50}$ and an equimolar concentration of 3.4 $\mu$M are shown in Figures 6 and 8, respectively.

**Western Blot Analysis.** To further verify the results seen in the ICC analysis, Western blot analysis was carried out on HaCaT cells treated for 9 and 48 h. At equimolar concentrations, neither naproxen nor Nap-DTH significantly altered the PCNA levels at 9 and 48 h whereas dithranol significantly downregulated PCNA levels at 48 h compared to the control ($p < 0.01$) (Figure 9). Figure 10 shows that, at a high concentration ($IC_{50}$), Nap-DTH and naproxen both led to a significant induction of caspase-3 expression after 9 and 48 h of exposure. However, when naproxen was used at 3.4 $\mu$M, there was no significant difference in caspase-3 expression compared to the control.

When dosed at $IC_{50}$, naproxen and Nap-DTH elevated levels of total COX-2 in HaCaT cells at 9 and 48 h, with high-dose naproxen showing the greatest (236% ± 21%) COX-2 upregulation among all treatment groups (Figure 11). In contrast, there was no significant difference in COX-2 levels when equimolar concentrations were used. For the 1:1 mixture, an upregulation of COX-2 was observed, although this change was not significantly different compared to the vehicle control. Treatment with dithranol alone led to significant upregulation of COX-2 (136% ± 31%) even with 9 h treatment ($p < 0.01$).

## DISCUSSION

**Cell Proliferation and Cytotoxicity.** Since it is a prerequisite that Nap-DTH releases its parent compounds *in situ* in order to exert therapeutic effects, a 1:1 mixture of the parent compounds (naproxen and dithranol) was used as a positive control in this study to mimic the complete liberation of Nap-DTH into the parent moieties. Naproxen exhibited the least inhibition on HaCaT proliferation compared to the other treatments. This is expected as nonsteroidal anti-inflammatory drugs (NSAIDs) are not known to inhibit cellular proliferation generally. In contrast, dithranol, a well-known antiproliferative drug, showed much lower IC values, demonstrating effective keratinocyte growth inhibition as predicted.[12] The data also demonstrated that the codrug, Nap-DTH, exerts a smaller inhibitory effect on HaCaT proliferation than dithranol. This is advantageous as the codrug is designed to remain inactive until the labile ester group is hydrolyzed *in situ*. The IC values for the 1:1 mixture indicate that the antiproliferative effect of the mixture was similar to that of dithranol. Taken together, these findings suggest that the codrug, in itself, has low therapeutic activity, but can confer a considerable antiproliferative effect upon hydrolysis to liberate the parent compounds.

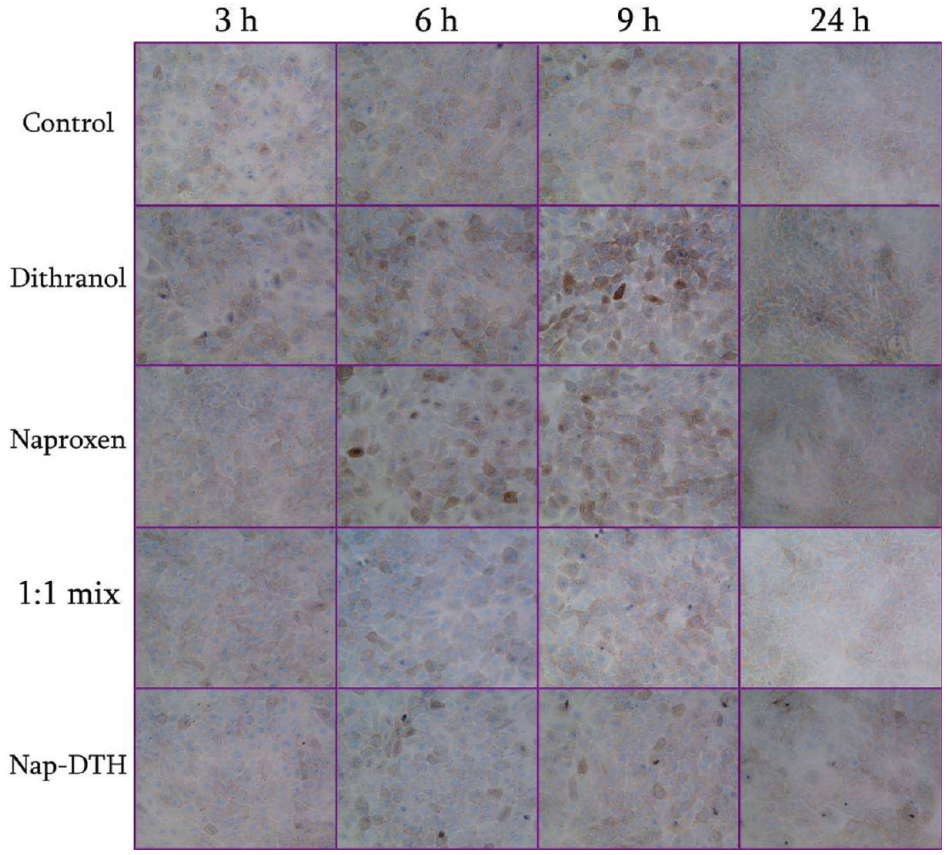


**Figure 6.** Representative images of ICC assay to detect COX-2 in HaCaT keratinocytes after incubation with different drug treatments using their respective $IC_{50}$ for 3, 6, 9, and 24 h: dithranol ($IC_{50}$ = 3.6 $\mu$M); naproxen ($IC_{50}$ = 2.5 mM); 1:1 mixture of naproxen:dithranol ($IC_{50}$ = 3.4 $\mu$M) and Nap-DTH ($IC_{50}$ = 52.7 $\mu$M), compare against the vehicle control. The control contained the vehicle (0.5% DMSO, 10% FBS in DMEM) only (original magnification ×200).

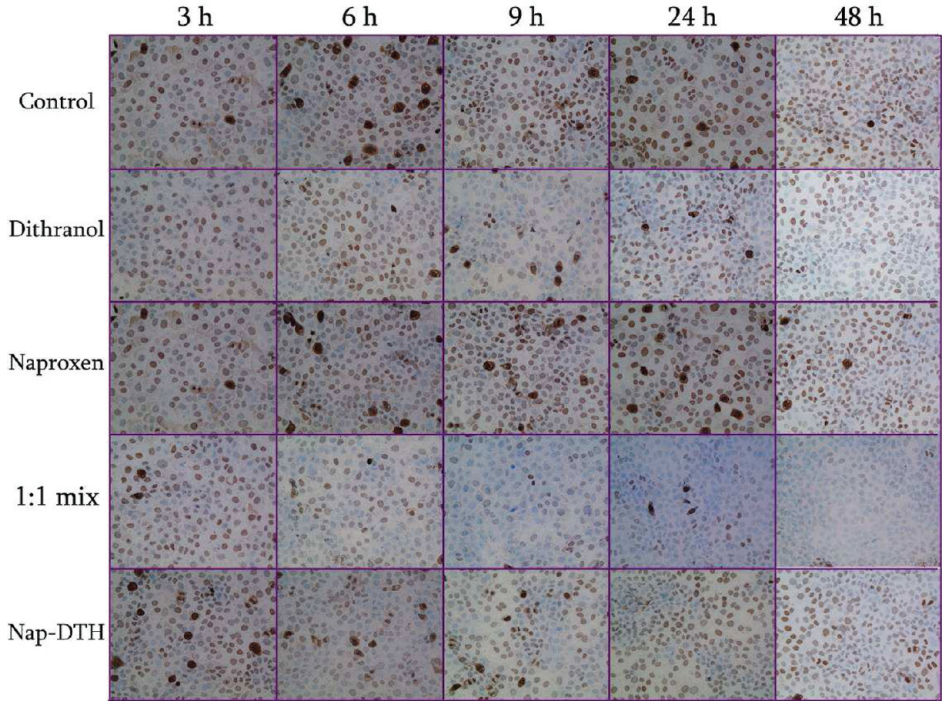


**Figure 7.** Representative images of ICC assay to detect Ki-67 in HaCaT keratinocytes at 3, 6, 9, 24, and 48 h after incubation in the presence or absence of an equimolar (3.4 $\mu$M) concentration of dithranol, naproxen, 1:1 mixture of naproxen:dithranol and Nap-DTH (original magnification ×200).

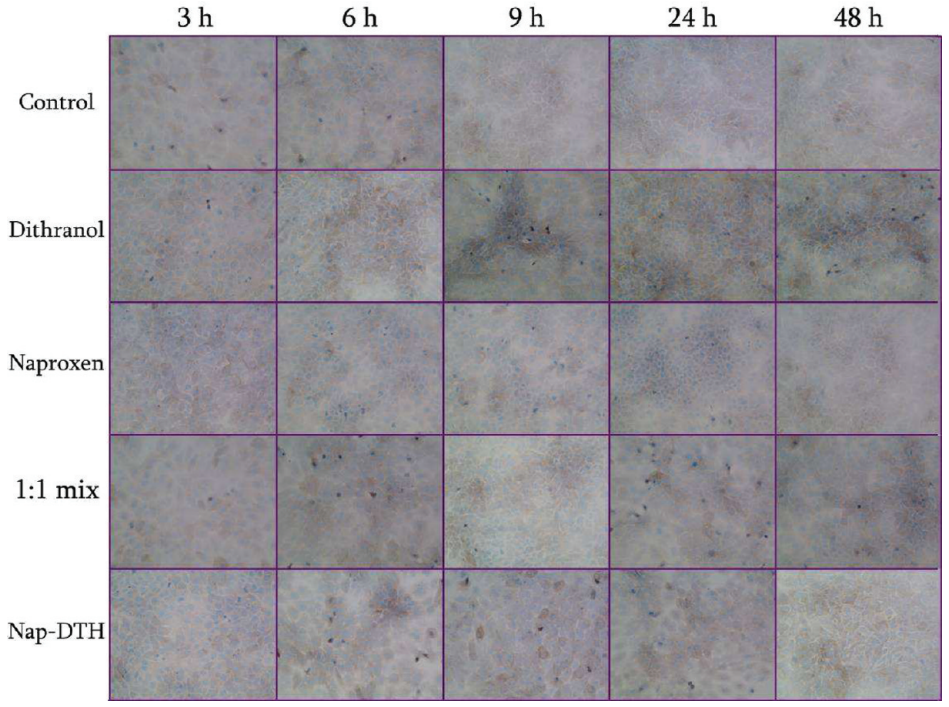


**Figure 8.** Representative images of ICC assay to detect COX-2 in HaCaT keratinocytes at 3, 6, 9, 24, and 48 h after incubation in the presence or absence of an equimolar (3.4 $\mu$M) concentration of dithranol, naproxen, 1:1 mixture of naproxen:dithranol and Nap-DTH (original magnification ×200).

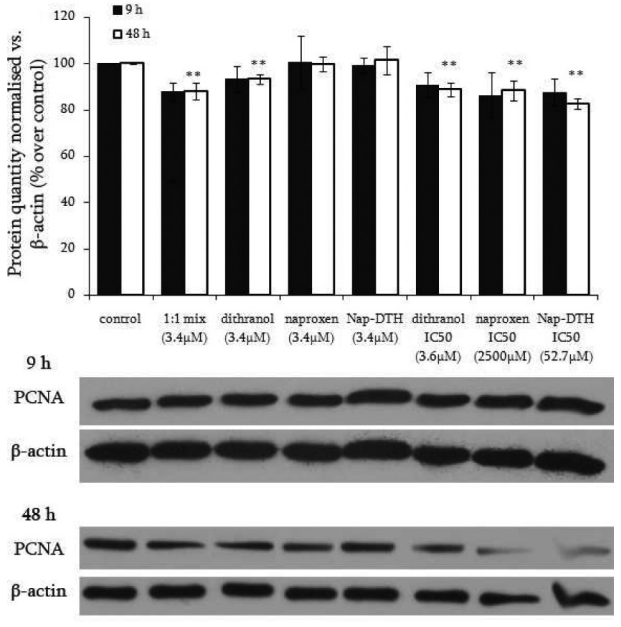


**Figure 9.** PCNA expression analyzed by Western blot. HaCaT cells were treated in the presence or absence of 1:1 mixture naproxen: dithranol, dithranol, naproxen, Nap-DTH (at 3.4 $\mu$M and at their $IC_{50}$ concentration) for 9 and 48 h. The histogram represents numerical data of PCNA normalized against $\beta$-actin (mean ± SD, $n$ = 3), and controls were adjusted to 100%. *$p$ < 0.05 and **$p$ < 0.01 indicate significant differences compared with the control.

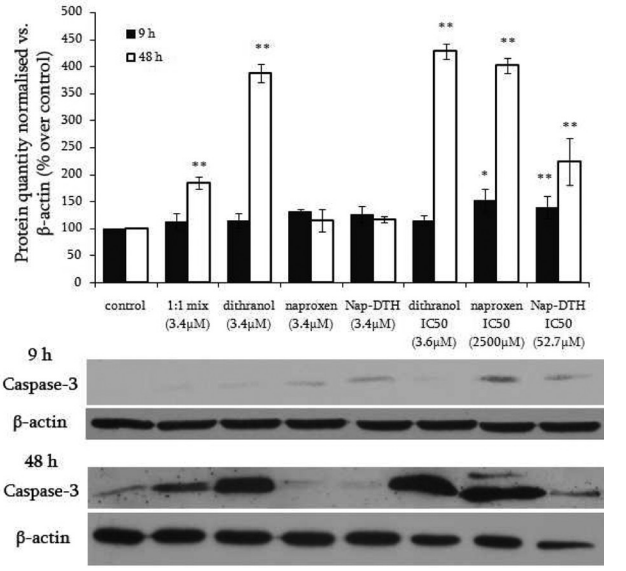


**Figure 10.** Cleaved caspase-3 expression analyzed by Western blot. HaCaT cells were treated in the presence or absence of 1:1 mixture of naproxen:dithranol, dithranol, naproxen, Nap-DTH (at 3.4 $\mu$M and at their $IC_{50}$ concentration) for 9 and 48 h. The histogram represents numerical data of cleaved caspase-3 normalized against $\beta$-actin (mean ± SD, $n$ = 3), and controls were adjusted to 100%. *$p$ < 0.05 and **$p$ < 0.01 indicate significant differences compared with the control.

To find out if the inhibition of keratinocyte growth derived from the MTT data was due to the antiproliferation effects of the compounds or cell death associated with plasma membrane damage, further experiments were performed where cell cytotoxicty was assessed on the basis of LDH leakage from the cells into the culture medium. The higher level of LDH release from both Nap-DTH and naproxen could be explained by the fact that the IC values for both were much higher compared to dithranol and

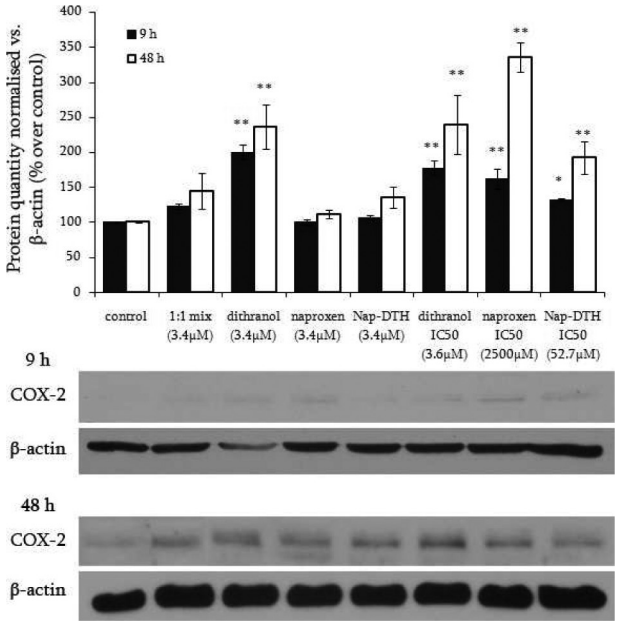


**Figure 11.** Analysis of COX-2 protein expression by Western blot. HaCaT cells were treated in the presence or absence of 1:1 mixture of naproxen:dithranol, dithranol, naproxen, Nap-DTH (at 3.4 $\mu$M and at their respective $IC_{50}$) for 9 and 48 h. The histogram represents numerical data of COX-2 normalized against $\beta$-actin (mean $\pm$ SD, $n = 3$), and controls were adjusted to 100%. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences compared with the control.

the 1:1 mixture. Therefore, greater cytotoxic effects would be expected by virtue of the higher concentrations of these compounds used. The significant LDH release from dithranol at 72 h suggests that the cytotoxicity of dithranol on HaCaT was not instantaneous. The LDH data further suggest that the cytotoxic effect of Nap-DTH was instantaneous as cell membrane damage occurred in the early phase of the LDH assay (within 24 h), whereby LDH released was the highest. Thereafter, there was much less but steady LDH release. Also, the data suggest that cytotoxicity of dithranol and naproxen may be reduced by the Nap-DTH codrug design.

There is no single assay that is capable of explicitly verifying cell viability and proliferation, thus flow cytometric analysis using double staining procedure was also carried out. The flow cytometric data demonstrated that the 1:1 mixture was associated with the lowest cytotoxicity at 48 and 72 h. This corresponded well with the LDH assay. The flow cytometric data further supported the observations derived from the LDH assays, i.e. the cytotoxic effect of naproxen was more latent, that of Nap-DTH was more immediate, and dithranol was more toxic to HaCaT cells than the 1:1 mixture even though their IC values were very similar.

In terms of antiproliferative activity, the codrug is assumed to be therapeutically inactive until the parent compounds are liberated *in situ*. Thus, it is not surprising that Nap-DTH showed no significant reduction in cell growth. Nonsteroidal anti-inflammatory drugs (NSAID) are known for their anti-inflammatory activities via the inhibition of COX. An increasing body of evidence suggests that NSAIDs can exert antiproliferation effects on tumor cells at concentrations 10- to 100-fold higher than those required for COX inhibition. It is thought that the mechanism for such effects involves the induction of apoptotic cell death in colon tumor cells,[21,22] although such effects have not been reported in human keratinocytes. The reduction in cell numbers observed in the MTT assay may reflect cell death through this mechanism rather than growth inhibition, since the flow cytometric analysis showed no significant cell growth inhibition in the naproxen-treated cells. In contrast, the antiproliferative agent, dithranol, showed growth inhibition. Moreover, the 1:1 mixture demonstrated a greater antiproliferation effect than dithranol alone. Thus, it can be surmised that the 1:1 mixture of naproxen:dithranol, when liberated from Nap-DTH *in situ*, would provide a greater antiproliferative effect on hyperproliferative keratinocytes in psoriatic skin than dithranol alone. This enhancement may be explained by stabilization of dithranol by the acidic naproxen, as dithranol is more stable in an acidic environment.[23]

**Immunocytochemical Analysis.** To investigate the potential anti-inflammatory and antiproliferative effects of Nap-DTH, HaCaT cells were treated with the codrug and its parent compounds, followed by ICC analysis to quantify the expression of cellular markers of proliferation (Ki-67) inflammation (COX-2). At $IC_{50}$, dithanol significantly reduced the level of Ki-67 compared with the control after 9 h of treatment, albeit to a lesser extent than the 1:1 mixture, despite the similar $IC_{50}$ for the two treatments. Naproxen did not significantly downregulate Ki-67 expression until the cells had been treated for 24 h, but the reduction could be due to the toxic effects of high-dose naproxen, which may cause necrosis and apoptosis.[21] In the case of Nap-DTH, a slight reduction in Ki-67 expression suggests that the codrug itself has low antiproliferative activity. In fact, the observed reduction cannot be confidently and wholly attributed to Nap-DTH, since the codrug is expected to degrade in HaCaT cells, liberating the antiproliferative parent compound, dithranol. In terms of COX-2 expression, dithranol treatment at $IC_{50}$ resulted in a noticeable upregulation of COX-2 6 h after incubation, compared to the vehicle control and other drug treatments. This is as expected as dithranol is known to induce local inflammation.[24] Naproxen is a COX inhibitor which in theory should reduce COX-2 expression. However, this anticipated effect was not observed when naproxen was dosed at its $IC_{50}$. On the contrary, an elevated COX-2 level was observed. The induction of COX-2 is likely to be due to the cytotoxic effect of naproxen at such a high concentration, which concurs with the results of the MTT and flow cytometric analyses (Figure 4). This toxic stimulus could induce inflammation and COX-2 expression. For Nap-DTH, no significant upregulation of COX-2 was observed, indicating that the codrug itself did not induce an inflammatory response even at its $IC_{50}$ concentration. A slight knockdown was observed after 24 h treatment. This could be due to partial hydrolysis of the codrug, leading to the liberation of naproxen. Unlike directly administered naproxen, naproxen liberated from the codrug was able to exert its anti-inflammatory effect without inducing inflammation due to the low dose used. Additionally, treatment with the 1:1 mixture resulted in the greatest reduction in COX-2 levels. These results strongly suggest that naproxen, when administered as Nap-DTH, would reduce inflammation that arises from dithranol as well as from the underlying pathophysiology of psoriasis itself.

In order to compare the cellular effects of the treatments, experiments using an equimolar concentration (3.4 $\mu$M) of each treatment were also carried out (Figure 7 and Figure 8 for Ki-67 and COX-2 respectively). The lowest $IC_{50}$ value was used in this case, which was the $IC_{50}$ of the 1:1 mixture, as this concentration

would be the least likely to cause unnecessary cytotoxic effects. To compensate for the lower drug concentration used, the treatment time was extended to 48 h. For naproxen, the downregulation of Ki-67, seen at $IC_{50}$ (2.5 mM), was not observed when naproxen was used at 3.4 $\mu$M. ICC also showed no significant difference in the expression of Ki-67 compared to the control, even at 48 h, when naproxen was used at this concentration (Figure 7). On the other hand, COX-2 levels were downregulated compared to the control (Figure 8). This was expected, since naproxen is used clinically for its ability to inhibit COX. There is evidence that, when used at a low dose, naproxen exerts an anti-inflammatory effect by the reduction of COX-2 level, but not an antiproliferative effect.[25] At equimolar concentrations, both Ki-67 and COX-2 levels in cells treated with Nap-DTH were similar to the control, further suggesting that the codrug is biologically inert. On the other hand, dithranol and the 1:1 mixture showed an inhibition of HaCaT proliferation, but the 1:1 mixture showed an enhanced effect with a greater reduction in Ki-67 level in spite of the equimolar concentrations. This again suggests that the presence of the acidic naproxen from Nap-DTH could stabilize the active dithranol. In terms of inflammatory response, at equimolar concentrations, both dithranol and the 1:1 mixture showed an upregulation of COX-2 compared to the control (Figure 8). However, when dithranol was dosed alone, a significantly higher level of COX-2 staining was seen at 9 h, whereas the 1:1 mixture took 24 h for COX-2 induction to be noticeable. The induction of COX-2 was less drastic in the 1:1 mixture, which suggests that the additional use of an equimolar concentration of naproxen could potentially minimize the inflammatory stimuli resulting from dithranol use.

**Western Blot Analysis.** PCNA, a cellular proliferation marker, was used for Western blot analysis to compare cellular proliferation after drug treatment on HaCaT keratinocytes (Figure 9). At equimolar concentrations, neither naproxen nor Nap-DTH significantly altered the PCNA levels at 9 and 48 h whereas dithranol significantly downregulated PCNA levels at 48 h compared to the control ($p < 0.01$). Furthermore, the 1:1 mixture at 3.4 $\mu$M, which mimics *in situ* Nap-DTH dissociation, showed approximately 2-fold reduction in PCNA level compared to dithranol. This further suggests that the addition of naproxen may enhance the antiproliferative effect of dithranol, perhaps through stabilization of the latter as discussed above.

In order to determine if the reduction of proliferative cells seen was due to induction of apoptosis, Western blot analysis using cleaved caspase-3 was carried out (Figure 10). Caspases are proapoptotic enzymes,[26] with caspase-3 being responsible for the proteolytic cleavage of many key proteins during apoptosis. Cleaved caspase-3 is the functionally active form of the enzyme, and so its cellular levels can be used to determine the progression of apoptosis. It is clear that Nap-DTH and naproxen, when used at a high concentration ($IC_{50}$), both led to a significant induction of caspase-3 expression after 9 and 48 h of exposure. This finding correlates well with other studies.[27,28] Hughes et al.[27] also found apoptosis induction when high dose naproxen (2.5 and 7.5 mM) was used, and suggested that the cell death and growth inhibition thus induced be harnessed to treat gastrointestinal cancers. However, such effects have not been demonstrated at lower concentrations where NSAIDs are used for their anti-inflammatory activities. When naproxen was used at 3.4 $\mu$M, there was no significant difference in caspase-3 expression compared to the control. The significant induction of caspase-3 expression with high-dose Nap-DTH and naproxen, together with the cytotoxic effects demonstrated in Figure 3, suggests that at high dose Nap-DTH could lead to induction of both necrotic and apoptotic cell death, hence the reduction in cell viability shown in Figure 4. Dithranol also showed a highly significant reduction in cleaved caspase-3 expression after 48 h treatment. Recent evidence has suggested that dithranol may induce apoptosis in keratinocytes by targeting the mitochondria.[29] The authors of the study reported an upregulation of caspase-3 by 600% in HaCaT cells when 5 $\mu$M of dithranol was used compared to the vehicle control, a result which is comparable to our finding where 3.4 $\mu$M of dithranol resulted in an upregulation of caspase-3 by 287% $\pm$ 17%. The 1:1 mixture also induced apoptosis but to a lesser extent than dithranol with 85% $\pm$ 11% at 48 h. This suggests that the antiproliferative effect seen with the 1:1 mixture of naproxen: dithranol was probably due to both a reduction in the growth rate of keratinocytes as well as the induction of apoptotic cell death. However, it is likely that the inhibition of cell growth plays a bigger role in the antiproliferative effect of the 1:1 mixture on HaCaT cells.

Western blot analysis showed that, in routine culture or after exposure to the vehicle control (containing 0.5% DMSO), HaCaT cells expressed low levels of COX-2 (Figure 11). This is in agreement with other studies employing HaCaT cells.[10,20] Both naproxen and Nap-DTH, when used at a high concentration, demonstrated an elevated level of total COX-2 at both 9 and 48 h. This correlates well with the ICC analysis (Figure 6), where naproxen at its $IC_{50}$ caused high COX-2 staining compared to dithranol. COX-2 levels were upregulated when high-dose Nap-DTH was used, but even at 48 h, the level of COX-2 expression was still 48% lower than the level of dithranol. This corroborates the earlier postulation that the codrug is essentially inert. It also implies that a higher concentration of Nap-DTH could be applied *in situ* without causing significant damage to tissues. This would make more codrug available for hydrolysis in the skin to liberate a sufficient amount of parent compounds to exert their therapeutic effects. When HaCaT cells were treated with the 1:1 mixture, a slight upregulation of COX-2 was observed ($p > 0.05$); whereas dithranol alone showed significant upregulation. This further supports that the coadministration of naproxen with dithranol reduces the proinflammatory effect of dithranol while it simultaneously enhances its antiproliferative effect.

## CONCLUSION

Our previous study has shown that the codrug Nap-DTH not only improves the delivery of naproxen and dithranol but also reduces the unwanted skin/clothes-staining effect of the parent moiety, which is a major issue concerning the use of dithranol. The current study has focused on the therapeutic and cytotoxic effects of the codrug, and shown that Nap-DTH itself is inert, but when fully liberated, exhibited the highest antiproliferation and anti-inflammatory effects with the lowest cytotoxic effects. The current work also suggests that inhibition of cell growth plays a more important role in the antiproliferation effect than the induction of apoptotic cell death with the use of Nap-DTH. It could also be inferred that dithranol dose-sparing can be achieved with the codrug Nap-DTH, and that this would reduce the potential adverse effects of the parent compounds. These findings strongly support our postulation that naproxen can enhance the stability of dithranol. The antiproliferative action from the liberated dithranol would complement the anti-inflammatory effect of the liberated naproxen to provide a dual therapeutic

action *in situ*. Therefore, the codrug, Nap-DTH, would be of immense value in the treatment of inflammatory hyperproliferative diseases like psoriasis.

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