Albendazole negatively regulates keratinocyte proliferation

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Clinical Perspectives

• Increased keratinocyte proliferation is one of the major pathological features of psoriasis.
• Topical application of Albendazole, a well-characterized anti-helmintic drug, inhibits keratinocyte proliferation and reduces the severity and extent of psoriasis-like skin lesions in mice.
• Albendazole could enter into the therapeutic armamentarium of psoriasis.

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Summary

Background. Increased keratinocyte proliferation occurs in the skin of psoriatic patients and is supposed to play a role in the pathogenesis of this disorder. Compounds interfering with keratinocyte proliferation could be useful in the management of psoriatic patients.

Aim. To investigate whether albendazole, an anti-helmintic drug that regulates epithelial cell function in various systems, inhibits keratinocyte proliferation in models of psoriasis.

Methods. Aldara-treated mice received daily topical application of albendazole. Keratinocyte proliferation and keratin (K) 6 and K16 expression were evaluated by immunohistochemistry and Western blotting and inflammatory cells/mediators were analyzed by immunohistochemistry and real-time PCR. In human keratinocytes (HEKa and HaCaT) treated with albendazole, cell cycle and proliferation, keratins and cell cycle-associated factors were evaluated by flow cytometry, colorimetric assay and Western blotting respectively.

Results. Aldara-treated mice given albendazole exhibited reduced epidermal thickness, decreased number of proliferating keratinocytes and K6/K16 expression. Reduction of CD3- and Ly6G-positive cells in the skin of albendazole-treated mice associated with inhibition of IL-6, TNF-α, IL-1β, IL-17A, IL36, CCL17, CXCL1, CXCL2 and CXCL5 expression. Treatment of keratinocytes with albendazole reduced K6/K16 expression and reversibly inhibited cell growth by promoting accumulation of cells in S-phase. This phenomenon was accompanied by down-regulation of CDC25A, a phosphatase regulating progression of cell cycle through S phase, and PKR-dependent hyper-phosphorylation of eIF2α, an inhibitor of CDC25 translation. In Aldara-treated mice, albendazole activated PKR, enhanced eIF2α phosphorylation and reduced CDC25A expression.

Conclusions. Data show that albendazole inhibits keratinocyte proliferation and exerts therapeutic effect in a murine model of psoriasis.

Keywords
Albendazole; benzimidazole, drug repurposing/repositioning; psoriasis; keratinocyte.
INTRODUCTION

Psoriasis is one of the most common chronic inflammatory disorders, affecting approximately 2-3% of the population worldwide (1). Well-demarcated, erythematous, scaly plaques, which can involve any part of the skin, but more commonly the extensor surfaces and the scalp, are typical lesions of the disease. Histologically, psoriasis is characterized by keratinocyte hyper-proliferation with parakeratosis and elongation or rete ridges, increased angiogenesis, and dermal infiltration with many inflammatory cells, such as macrophages, dendritic cells, T cells, and neutrophils(2-4).

The aetiology of psoriasis remains unknown even though accumulating evidence suggests that psoriasis develops in genetically predisposed individuals as a result of the action of various environmental factors, which trigger innate and adaptive immune responses(5, 6). As a consequence, many effector cytokines produced in the psoriatic skin [e.g. tumour necrosis factor (TNF), interleukin (IL)-6, IL-17, IL-21, IL-22, and IL-23] stimulate keratinocyte activation/proliferation(7-11). This sequence of events is accompanied by secretion of chemoattractants, which promote recruitment of other inflammatory cells, such as neutrophils, into the affected skin thereby leading to the amplification of the detrimental inflammatory response(3).

In line with such a hypothesis is the observation that compounds targeting the above cytokines are useful for inducing and maintaining remission in psoriatic patients(12-16). Unfortunately however, not all the patients respond to these drugs and in some patients treatment must be discontinued due to the development of adverse events or other immune-mediated diseases(17-19). Thus, the development of novel compounds targeting the key pathogenic events in psoriasis is worth pursuing.

Studies in murine models of psoriasis have also shown that primary defects of keratinocytes could be sufficient to promote epidermal hyper-proliferation and psoriasis-associated immune-
inflammatory reactions, raising the possibility that drugs interfering with keratinocyte function could be useful in the management of psoriatic patients (20-22).

Since the development of new drugs is a very lengthy and costly process, drug repositioning, defined as identifying novel indications for existing drugs, has recently become a good strategy for overcoming the limitations of traditional methods.

Albendazole is a well-characterized benzimidazole derivative, initially developed as an anti-helmintic drug. Albendazole-mediated biologic effects rely on inhibition of tubulin polymerization and blockage of glucose uptake (23-25). Recent experimental studies have shown that albendazole has also anti-tumoral properties in many epithelial cancers (e.g. hepatocellular carcinoma, cutaneous squamous cell carcinoma, and colorectal carcinoma) mainly depending on its ability to interfere with cancer cell growth (26-28). Based upon these observations, we hypothesized that albendazole can also regulate keratinocyte proliferation/activation and exert protective effects in psoriasis. The aim of this study was to investigate whether albendazole inhibits psoriatic lesions in an in vivo model of disease and to dissect the mechanisms by which the drug interferes with keratinocyte proliferation.
MATERIALS AND METHODS

Mice studies

C57bl/6 mice (male, 8-10 weeks of age) were purchased from Charles River and hosted in the conventional animal facility at the University of Rome “Tor Vergata”. Animal Ethics Committee, according to Italian legislation on animal procedures, approved all the animal experiments (N° 597/2016-PR). All reagents were from Sigma-Aldrich (Milan, Italy) unless specified. To induce a psoriasis-like skin inflammation, shaved mice were treated daily with 62.5 mg of commercially available Aldara cream (Meda, Solda, Sweden) on the back skin for 4 consecutive days. Vaseline cream was used as internal control (vehicle). Methyl 5-propylthio-2-benzimidazolecarbamate (albendazole) (30μg/mouse), resuspended in 100 μl of propylene glycol, was daily applied on the shaved back of Aldara-treated mice in the same skin area, starting 12 hours after the Aldara administration. Control mice were daily treated with a similar daily amount of propylene glycol 12 hours after the Aldara treatment. All animals were assessed for the severity of the psoriasis-like skin condition at day 4, using three elements of the Psoriasis Area Severity Index (PASI), assigning a score of 0 – 4 (0: none; 1: mild; 2: moderate; 3: severe; 4: very severe) for each of the parameters erythema, scaling and induration. This analysis was performed in at least 4 fields per section of all the skin samples.

Histopathological analysis and immunohistochemistry

Cryosections of mouse skin samples were stained with hematoxylin and eosin (H&E), and epidermal thickness was evaluated by measuring the average interfollicular distance from the basal lamina to the bottom of the stratum corneum(8, 29). Mouse sections were also stained with rat anti-ki67 antibody (Dako, Milan, Italy) followed by a secondary antibody anti-rat conjugated to horseradish peroxidase (Dako). Likewise, sections were stained with rabbit anti-CD3 (SP7) (Abcam, Cambridge, UK), rat anti-Ly6G (BD Pharmingen, San Jose, CA, USA), rabbit anti-Cleaved Caspase 3 (Cell Signaling Technology, Danvers, MA, USA) and positive cells were
visualized using MACH4 Universal HRP- Polymer kit with DAB (Biocare Medical, Pacheco, CA). Isotype control sections were prepared under identical immunohistochemical conditions, replacing the primary antibody with a purified control isotype (IgG). Positive cells were analyzed by LEICA DMI4000 B microscope using LEICA application suite software (V4.6.2). The percentage of skin area covered by positive cells was evaluated using Image J (NIH) software.

Cell cultures

The human immortalized keratinocyte cell line, HaCaT, was obtained from the Creative Bioarray (Shirley, NY, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.05% bovine serum albumin (BSA), glutamine (4 mM) and 0.05 mg/ml gentamycin. Human Epidermal Keratinocyte adult (HEKa) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dermal Cell Basal Medium supplemented with 0.4% of Bovine Pituitary Extract (BPE), TGF-α (0.5ng/ml), L-glutamine (6 mM), Hydrocortisone Hemisuccinate (100ng/ml), Insulin (5mg/ml), Epinephrine (1 mM) and Apo-Transferrin (5mg/ml) (all reagents from ATCC). To inhibit p-double-stranded RNA-activated protein kinase (PKR), HaCaT cells were transfected with either scrambled-selective short interfering RNA (siRNA) or PKR-siRNA (both used at 100 nM) for 24 h and then incubated both for 30’ in presence or absence of albendazole (30nM). In parallel experiments, HaCaT and HeKa cells were incubated with TNF-α (25ng/ml) for 4 hours in presence or absence of albendazole (30nM). Additionally, HaCaT were treated with PKR-siRNA and 24 hour later treated with TNF-α (25ng/ml) in presence or absence of albendazole for 4 hours. HaCaT were also incubated with Salubrinal (40μM) for 24 hours.

Analysis of cell death, cell proliferation and cell cycle distribution

To evaluate whether albendazole affects cell death, HaCaT cells were treated with or without increasing concentrations of the compound (30 nM-1μM) for 24h and 48h and then
assessed by flow cytometry. After culture, cells were collected, washed twice in PBS, stained with FITC-annexin V (AV, 1:100 final dilution, Immunotools, Friesoythe, Germany) according to the manufacturer’s instructions and incubated with 5 μg/ml propidium iodide (PI) for 30 min at 4°C. Fluorescence was measured using the FL-1 and FL-3 channels of Gallios (Beckman Coulter, Milan, Italy) flow cytometer. Viable cells were considered as AV-/PI- cells, apoptotic cells as AV+/PI- cells, while secondary necrotic cells were characterized by AV+/PI+ positive staining. Cell proliferation was assessed by using a commercially available 5-bromodeoxyuridine (BrdU) assay kit (Roche Diagnostic GmbH, Mannheim, Germany). Cells were incubated with 30nM of albendazole and BrdU was added to the cell cultures 6 hours before the end of the 24h and 48h treatments. BrdU-positive cells were evaluated by ELISA. To determine whether the anti-proliferative effect of albendazole was reversible, HaCaT cells were either left untreated or treated with albendazole for 24 hours, then washed with PBS and cultured with fresh medium for further 24 hours. Cell proliferation was then assessed by 5-bromodeoxyuridine (BrdU) assay. For analysis of cell cycle distribution, HaCaT and HEKa were either left untreated or treated with albendazole. After 24 hours, cells were pulsed with 10 mol/L BrdU for 60 minutes, fixed in 70% cold ethanol, and stored at 20°C for at least 3 hours. Cells were then denatured in 2 mol/L HCl, and stained with anti-BrdU monoclonal antibody (Roche Diagnostics) followed by fluorescein isothiocyanate–conjugated secondary anti-mouse IgG (Molecular Probes, Milan, Italy). After staining with PI, cells were analyzed by flow cytometry.

RNA extraction and Real-time PCR

Total RNA was isolated from skin biopsies using TRIzol reagent and from cells using PureLink® Purification technology (Thermo Fisher Scientific). A constant amount of RNA (1μg/sample) was retrotranscribed into complementary DNA (cDNA). Reverse transcription was performed with Oligo(dT) primers and with M-MLV-reverse transcriptase (Thermo Fisher Scientific). Real-time PCR was performed using TaqMan gene assays for hIL-36 (α,β,γ) (Thermo Fisher Scientific).
Fisher Scientific) and IQ SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy) for the other genes.

Primer sequences were as follows: mIL-6 forward 5’- AGCCAGAGTCCCTTCAGAGAG -3’, reverse 5’- GATGGCTTTGTCCTTAGCC -3’; mTNF-α forward 5’- ACCCTCACAATCAGATCATC, reverse 5’- GATGGCTTTGTCCTTAGCC -3’; mIL-1β forward 5’- TCAGGCAGGCAGTATCCT -3’, reverse 5’- CTAATGGGAACGTCACACACC -3’; mIL-17A forward 5’- TCAGACTACCTCAACCGTTC -3’, reverse 5’- TTCAGGACCAGATCTCTTG -3’; mCCL17 forward 5’- TTTCTGACTGTCCAGGGCAA -3’, reverse 5’- TTTGTGTTTCGCTGTAGTGC -3’; mCXCL1 forward 5’- GCTGGGATTCCACCTCAAGAAC -3’, reverse 5’- AAGGGAGCTTCAGGGTCAAG -3’; mCXCL2 forward 5’- CGCTGTCAATGCGCTGAAGAC -3’, reverse 5’- ACACTCAAGCTCTGGATGTTCTTG -3’; mCXCL5 forward 5’- CTGCCCTTCTCAGTCTAG -3’, reverse 5’- GGATCCAGACAGACCTCTCTTC -3’; hK6A forward 5’- TGAAGGAGTACCAGGAACTC -3’, reverse 5’- CACCACAGAGATTTGACTG -3’; hK16 forward 5’- AAGACTACGCCCCTCCTTC -3’, reverse 5’- CATTTCGTACTTTGTCCTG -3’; hCDC25A forward 5’- GTACAAGAGAGAGGGAAGAGC -3’, reverse 5’- GATGCCAGGGAT AAAGACTG -3’; hIL-8 forward 5’- AGGAACCATCTCAAGACGTGT -3’, reverse 5’- CCACTCCTCATTCAACCTCATC -3’; hIL-6 forward 5’- CCACTCACCTCTCAGAAAC -3’, reverse 5’- CCACTCACCTCTCAGAC -3’; hIL-1β forward 5’- AGAATGACCTGAGCACCCTTC -3’, reverse 5’- GCCTCTTGGCCTTCCCACAC -3’; hIL-1β forward 5’- AGAATGACCTGAGCACCCTTC -3’, reverse 5’- GCCTCTTGGCCTTCCCACAC -3’; hCCL5 forward 5’- TGCTGCTTTGCTTACTTG -3’, reverse 5’- CCAGAACCTCTTCTCTCT -3’. RNA expression was calculated relative to the housekeeping human/mouse β-actin gene (forward 5’- AGAGATGACCCAGATCATGTTTTGAGACC -3’, reverse 5’- AGCCAGTCCAGAGCAGAG -3’) on the base of the ΔΔCt algorithm.
Western blotting

Cell and skin samples were lysed on ice in buffer containing 10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EthyleneDiamineTetraacetic Acid (EDTA), 0.2 mM Ethylene Glycol-bis (β-aminoethyl ether)-N,N,N',N'-Tetraacetic Acid (EGTA) and 0.5% Nonidet P40 supplemented with 1 mM dithiothreitol (DTT), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, and 1 mM NaF. Lysates were clarified by centrifugation and separated on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Blots were incubated with antibodies against K6A (1 : 1000 final dilution, Spring Bioscience, Pleasanton, CA, USA), K16 (1 : 1000 final dilution, Novus Biologicals, Littleton, CO, USA), cyclin-dependent kinase (CDK)2 (sc-6248), phosphorylated (p)-CDK2 (sc-28435), cell division cycle 25 (CDC25A) (sc-7389), CDC25B (sc-5619), CDC25C (sc-13138), eukaryotic initiation factor-2 α (eIF2α) (sc-11386), p-PKR-like ER resident kinase (PERK) (sc-31577), (1 : 500 final dilution, all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-eIF2α (Ser51) and Cleaved Caspase 3 (1 : 1000 final dilution, both from Cell Signaling), PKR (Thr446) (1:1000 final dilution, Thermo Fisher Scientific, Waltham, MA, USA), PKR (Affinity BioReagents, Dublin, OH, USA), p-general control non-derepressible 2 kinase (GCN2) (1:500 final dilution - Abcam) followed by a secondary antibody conjugated to horseradish peroxidase (Dako, Milan, Italy). After analysis, each blot was stripped and incubated with a mouse–anti-human monoclonal β-actin antibody (1 : 5000 final dilution, Sigma-Aldrich) to ascertain equivalent loading of the lanes.

Statistical analysis

Data were analyzed using the two-tailed Student's t-test for comparison between two groups or Mann Whitney U test. Significance was defined as P-values < 0.05.
RESULTS

Albendazole reduces severity of psoriasis-like lesions and keratinocyte proliferation

To investigate whether albendazole inhibits keratinocyte proliferation, we initially used a well-characterized model of epidermal hyper-proliferation and abnormal keratinocyte differentiation induced in mice by topical application of Aldara cream(30). Our previous time-course studies showed that mice given Aldara exhibited the more pronounced severe skin pathology at day 4 (31). Therefore, this time point was selected to evaluate the therapeutic effect of albendazole. In such a model, a daily topical application of albendazole, given 12 hours after Aldara treatment, decreased the severity and extent of psoriasis-like lesions in terms of erythema, scaling and skin thickening/induration with significant reduction of PASI score (Fig. 1A). H&E staining showed reduced epidermal thickness, parakeratosis and cellular infiltration in mice treated with albendazole (Fig. 1B). Similar effects were seen when analysis was performed at day 6 of Aldara treatment (Suppl. Fig 1A). The immunostaining of ki-67, a marker of cell proliferation, revealed less proliferative cells in the epidermal basal stratum of albendazole+Aldara-treated mice as compared to Aldara-treated mice (Fig. 1C). Moreover, albendazole treatment decreased expression of K6A and K16, two differentiation-specific epidermal keratins induced in hyper-proliferative keratinocytes(32, 33) (Fig. 1D). By Western blotting we also showed a reduced expression of cleaved caspase-3 following albendazole treatment clearly indicating the modulatory effect of the drug on keratinocytes is not secondary to induction of apoptosis or cell damage (Suppl. Fig 1B-C).

Albendazole reduces the infiltration of immune cells and production of inflammatory molecules in the skin of Aldara-treated mice

Next, we investigated whether the therapeutic effect of albendazole was paralleled by reduced infiltration of the skin with neutrophils and T cells, as these cells are supposed to amplify pathogenic signals in psoriasis(4, 34). Albendazole-treated mice exhibited reduced cutaneous infiltration of CD3- and Ly6G-expressing cells (Fig. 2A and Suppl. Fig.1D) and this associated with
significant decrease of inflammatory cytokines, such as IL-6, TNF-α, IL-1β, IL-17A, and IL-36(α,β,γ) and reduced expression of chemokines involved in the recruitment of neutrophils and T cells, such as CCL17, CXCL1, CXCL2 and CXCL5 (Fig. 2B).

We also examined whether the in vivo anti-proliferative effect of albendazole on keratinocytes was secondary to inhibition of production of immune cell-derived cytokines, which are known to stimulate keratinocyte growth. To this end, skin explants of Aldara-treated mice were cultured with or without albendazole for a short time (i.e. 6 hours) and the levels of K6A, K16, TNF-α and IL-6 transcripts were then analysed by real-time PCR. Albendazole reduced K6A and K16 RNA transcripts without changing IL-6 and TNF-α RNA expression (Fig. 3).

Albendazole reversibly blocks keratinocyte cell growth

The above findings suggest that albendazole targets primarily keratinocytes. To support this hypothesis, we evaluated the anti-proliferative effect of the compound in HaCaT cells, a non-tumorigenic monoclonal highly proliferating human keratinocyte line, which shares some features with psoriatic keratinocytes. In preliminary experiments, we showed that albendazole reduced cell viability at doses ranging from 125 nM to 1 μM (Fig. 4A). Therefore, the subsequent studies were conducted using 30 nM albendazole. HaCaT cells cultured with albendazole exhibited reduced protein expression of K6A and K16, and decreased growth, which was evident at both 24 and 48 hours (Fig. 4B-C). Such effects were fully reversible, because HaCaT cells proliferated regularly after removal of albendazole from the culture (Fig. 4D).

Keratinocytes accumulate in S-phase of the cell cycle following albendazole treatment

In subsequent studies, we assessed the effect of albendazole on cell-cycle progression in HaCaT cells. CDK complexes orchestrate the progression through S (Synthesis) and M (Mitosis) phases of cell cycle. These steps are precisely regulated by multiple events, including removal of
inhibitory phosphorylation on CDKs by CDC25 phosphatase family members(35). Specifically, CDC25B and CDC25C control the transition from G2 to M phase by primarily dephosphorylating CDK1, while CDC25A plays a more extensive role in assisting G1/S progression by CDK2 dephosphorylation(36, 37). The albendazole-induced cell growth inhibition was associated with accumulation of cells in S phase, reduction of CDC25A protein expression, and up-regulation of CDK2 phosphorylation, while protein levels of CDC25B and CDC25C remain unchanged (Fig. 5A-B). Similarly, HEKa accumulated in S-phase of cell cycle after albendazole treatment (Suppl. Fig. 2A-B). No significant change in CDC25A mRNA expression was seen in albendazole-treated cells (Fig. 5C), indicating that the inhibitory effect of the compound on CDC25 expression occurs at post-transcriptional level.

Albendazole promotes PKR-mediated eIF2α inactivation and inhibition of inflammatory molecules in keratinocytes.

Many forms of cellular stress, such as hypoxia, nutrient deprivation, DNA damage, misfolded protein accumulation and endoplasmic reticulum stress can cause cell cycle arrest through the phosphorylation on serine residue 51 of the α subunit of the eIF2α(38−40). This phenomenon is mediated by several up-stream kinases, such as PKR, PERK, GCN2 and results in the inhibition of translation machinery of several RNAs, with the downstream effect of suppressing protein expression of many regulators of cell cycle, including CDC25A(41). Therefore, we assessed whether reduction of CDC25A protein content and arrest of the cell cycle in the S-phase following albendazole treatment was associated with changes in the phosphorylation of eIF2α. Treatment of HaCaT cells with albendazole led to increased eIF2α phosphorylation, which was evident as early as 15 minutes following albendazole treatment and was maintained during the various time points of the experiment (Fig. 6A, upper blot). Hyper-phosphorylation of eIF2α and accumulation of the cells in S-phase of the cell cycle were documented in HaCaT after treatment with Salubrinal, a specific eIF2α inhibitor; addition of albendazole to these cultures caused no further change in eIF2α.
phosphorylation and in the number of cells accumulated in S-phase of the cell cycle (Suppl. Fig. 2C-D).

Time-course studies showed also that eIF2α phosphorylation was preceded by phosphorylation of PKR (Thr446) (Fig. 6A), while expression of phosphorylated PEKR and GCN2 remained unchanged (data not shown). Down-regulation of PKR expression with a specific siRNA prevented albendazole-mediated eIF2α phosphorylation (Suppl. Fig 2E and Fig. 6B) as well as abrogated the albendazole-mediated inhibitory effect on IL-6, IL-8, IL-1β, and CCL5 expression in TNFα-activated keratinocytes (Fig. 6C-D and Suppl. Fig 2F-G).

To translate these data in vivo, phosphorylation of eIF2α and PKR and expression of CDC25A were evaluated in the skin of Aldara-treated mice receiving or not albendazole. Albendazole enhanced phosphorylation of both eIF2α and PKR and this was associated with reduction of CDC25A protein expression (Fig. 7).
DISCUSSION

Enhanced keratinocyte proliferation is documented in the psoriatic skin and supposed to make a valid contribution to the pathogenesis of psoriasis\(^{42, 43}\). Therefore, compounds interfering with keratinocyte proliferation could be useful in the management of psoriatic patients\(^{44}\). Drug repurposing known also as drug repositioning or drug repromiling is an approach aimed at investigating whether drugs, which are already approved to treat pathologies, are safe and effective for treating other diseases. Drug repurposing offers significant benefits to the pharmaceutical industries as it overcomes most of the cost and time-consuming hurdles during the drug development process.

This study was undertaken to evaluate whether albendazole, a well-characterized anti-helmintic drug commonly used for the treatment of parenchymal neurocysticercosis, has the potential to inhibit keratinocyte proliferation and attenuate psoriasis-like skin lesions. Initial studies performed in a well-characterized mouse model of psoriasis induced by Aldara\(^{30, 31}\) showed that topical application of albendazole drastically reduced the severity and extent of skin lesions. Mice treated with albendazole exhibit reduced epidermal thickness, parakeratosis and rete ridges development associated with decreased number of proliferating keratinocytes in the basal layer of epidermis and downregulation of K6A and K16, two differentiation-specific epidermal markers overexpressed in psoriatic epidermis\(^{32, 33}\). Immunohistochemical analysis of skin sections revealed in the albendazole-treated mice reduced skin infiltration of CD3- and Ly6G-positive cells, which was associated with decreased expression of inflammatory mediators such as IL-6, TNF-\(\alpha\), IL-1\(\beta\), IL-17A, IL-36, CCL17, CXCL1, CXCL2 and CXCL5. To evaluate whether the inhibitory effect of albendazole on keratinocytes was secondary to the reduced production of such inflammatory cytokines, we cultured skin explants of Aldara-treated mice with albendazole for a short time and then RNA expression of keratinocyte markers and immune markers was evaluated by real-time PCR. Albendazole treatment reduced expression of K6A and K16 without modifying IL-6 and TNF-\(\alpha\) RNA transcripts, thus suggesting that the inhibitory effect of the drug on
keratinocytes precedes its modulatory action on immune cells. Consistently, albendazole inhibited proliferation of HaCaT cell line, a highly proliferating human keratinocyte cell line, at concentrations that were associated with no induction of cell death. Overall these data are in line with previous studies showing a negative effect of albendazole on other epithelial cell types and particularly epithelial cancer cells (27, 45).

The anti-proliferative effect of the drug was reversible and marked by accumulation of cells in S phase of cell cycle. This phenomenon was accompanied by reduced protein expression of CDC25A, a phosphatase that controls progression through S phase of cell cycle (46, 47). Inhibition of CDC25A by albendazole occurred at protein and not RNA level, thus implying a post-transcriptional control of CDC25A. Interestingly, treatment of keratinocytes with albendazole triggered phosphorylation of eIF2α, a key regulator of translation attenuation and suppressor of CDC25A protein expression (48) both in HaCaT and primary keratinocytes (HEKa). The intracellular target of albendazole and the exact molecular mechanism by which the drug regulates the cell cycle machinery in keratinocyte remain to be ascertained. It is known that albendazole inhibits tubulin polymerization and blockage of glucose uptake (23-25), two phenomena which could eventually induce cellular stress and promote activation of kinases involved in the control of cell cycle (39, 40). However, inhibition of tubulin polymerization should cause a block of cells in G2/M phase rather than S-phase of the cell cycle. It is thus conceivable that, at least in keratinocytes, at the specified concentrations albendazole does not act primarily as a microtubule inhibitor. Time-course studies showed that phosphorylation of eIF2α was preceded by phosphorylation of PKR while other up-stream kinases, such as PEKR and GCN2, were not affected by albendazole. We are confident that PKR can be involved in the phosphorylation/inactivation of eIF2α, as previous studies have shown that eIF2α is a major target of PKR (49), and data of the present work show that specific silencing of PKR prevented albendazole-mediated eIF2α phosphorylation and the production of inflammatory mediators.
involved in psoriasis pathogenesis. Consistently, Aldara-treated mice receiving albendazole had elevated levels of phosphorylated PKR and eIF2α and reduced levels of CDC25A in the skin.

In conclusion, our findings reveal a novel inhibitory action of albendazole on keratinocyte proliferation, which associates with a therapeutic effect in a murine model of psoriasis.
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Conflict of interest

Giovanni Monteleone has filed a patent related to the treatment of inflammatory bowel diseases with SMAD7 anti-sense oligonucleotides. The remaining authors declare no conflict of interest.

Author contribution

Davide Di Fusco: Conceptualization, Methodology, Investigation, Validation, Formal analysis Writing - Original Draft, Visualization; Carmine Stolfi: Conceptualization and Methodology; Antonio Di Grazia, Vincenzo Dinallo, Federica Laudisi, Irene Marafini and Alfredo Colantoni: Methodology, Investigation and Ivan Monteleone: Conceptualization, Methodology. Giovanni Monteleone: Project administration, Conceptualization, Writing - Original Draft, Supervision
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Figure Legends

Figure 1. Albendazole treatment reduces severity of psoriasis-like lesions and keratinocyte proliferation. A) C57Bl/6 mice treated with vehicle, Aldara cream and Aldara+albendazole for 4 consecutive days. Albendazole (30μg/mouse) was topically applied each day starting 12 hours after Aldara treatment. Photographs of representative shaved back skin of the mice are shown. Right inset shows the Psoriasis Area Severity Index (PASI) score (0=none; 1=mild; 2=moderate; 3=severe; 4=very severe, for each of the following parameters: erythema, scaling and induration) evaluated at day 4 in the untreated and treated mice. Values indicate mean ± SD of 3 experiments analysing in total 12 mice per group. (Vehicle-treated mice vs Aldara-treated mice; *P<0.05). (Aldara-treated mice vs Aldara+albendazole-treated mice; **P<0.05). B) Representative hematoxylin and eosin (H&E)-stained skin sections of mice treated as above. One of 3 separate experiments in which 12 mice/group were analyzed is shown. Epidermal thickness (right inset) was evaluated in H&E-stained skin sections of mice by measuring the distance from the basal lamina (black dashed line) to the bottom of the stratum corneum. This analysis was performed in at least 4 fields per section of all the skin samples. Data are expressed as mean ± SD of all experiments. (Vehicle-treated mice vs Aldara-treated mice; *P<0.05). (Aldara-treated mice vs Aldara+albendazole-treated mice; **P<0.05). C) Representative immunostaining for ki67 of skin sections of mice treated as indicated above. Staining with isotype IgG is also shown. Right inset indicates the percentage of skin area covered by ki67-positive cells. Values indicate mean ± SD. Aldara-treated mice vs Aldara+albendazole-treated mice *P<0.05. D) Total proteins extracted from the back skin of mice treated as above were analyzed for keratin (K) 6A and K16 expression by Western blotting. The blot is representative of 3 separate experiments in which similar results were obtained. β-actin was used as loading control.

Figure 2. Topically application of albendazole reduces inflammatory signals in the skin of psoriatic mice. A) Representative immunostaining for CD3+ and Ly6G+ cells in skin sections
taken from the back of psoriatic mice treated with Aldara or with Aldara+albendazole for 4 consecutive days. Albendazole (30μg) was topically applied each day starting 12 hours after Aldara treatment. Staining with isotype IgG is also shown. Right inset indicates the percentage of skin area covered by CD3+/Ly6G+ cells. Values indicate mean ± SD. (Aldara-treated mice vs Aldara+albendazole-treated mice; **P<0.05). B) Albendazole reduces IL-6, TNF-α, IL-1β, IL-17A, CCL17, IL-36 (α,β,γ), CXCL1, CXCL2 and CXCL5 mRNA expression in the skin of psoriatic mice treated as above. RNA transcripts were determined by Real-time PCR and normalized to β-actin. Data indicate mean ± SEM of three separate experiments in which 12 mice/group were included. (Vehicle-treated mice vs Aldara-treated mice; *P<0.05). (Aldara-treated mice vs Aldara+albendazole-treated mice; **P<0.05).

**Figure 3.** Ex-vivo short-term treatment of psoriatic skin explants with albendazole reduces expression of keratinocyte genes (i.e. K6A and K16) without affecting expression of inflammatory molecules (i.e. IL-6 and TNF-α). Murine skin samples taken from shaved C57bl/6 mice treated with Aldara cream for 2 consecutive days were cultured with or without albendazole (30μg) for 6h. RNA transcripts for K6A, K16, IL-6 and TNF-α were analyzed by Real-time PCR and normalized to β-actin. Data indicate mean ± SEM of three separate experiments: in each experiment at least 3 mice per group were considered. (Aldara-treated mice vs Aldara+albendazole-treated mice; *P<0.05).

**Figure 4.** Albendazole reversibly blocks keratinocyte proliferation. A) Representative dot plots showing the percentages of AV- and/or PI-positive HaCaT cells. Cells were treated with increasing concentrations of albendazole (ranging from 30 nM to 1 μM) for 24h and then assessed by flow cytometry analysis. Representative histograms (right panel) showing the percentage of HaCaT cell death treated as indicated above. Data are expressed as mean ± SEM of 3 separate experiments. Dimethyl sulfoxide (DMSO) and Staurosporine were included as internal controls. B) Treatment of
HaCaT cells with albendazole (30 nM) associates with reduction of keratin (K) 6A and K16 protein expression. Cells were stimulated for 24h with and without of albendazole and K6A and K16 were evaluated by Western blotting. β-actin was used as loading control. One of 3 representative experiments, in which similar results were obtained, is shown. C) Albendazole treatment reduces keratinocyte proliferation. Cells were incubated in the presence/absence of albendazole (30 nM) for 24h and 48h and 5-bromo-2-deoxyuridine (BrdU) was added to the cell cultures 6 hours before the end of the treatment. BrdU-positive cells were evaluated by colorimetric assay. Data are expressed as percentage of BrdU-incorporated cells over control (unstimulated) and indicate mean ± SEM of 3 separate experiments. Albendazole-treated cells vs unstimulated cells. *P<0.05. D) Albendazole (alb) reversibly blocks keratinocyte proliferation. HaCaT were incubated in the presence or absence of albendazole for 24h, then washed and cultured for further 24 h in fresh medium (M) in the absence of albendazole. BrdU was added to the cell cultures 6 hours before the end of the treatment and BrdU-positive cells were evaluated by colorimetric assay. Data are expressed as percentage of BrdU-incorporated cells over control and indicate mean ± SEM of 4 separate experiments. *P<0.05.

Figure 5. Albendazole induces keratinocytes to arrest in S-phase of the cell cycle. A) Cells were incubated in the presence or absence of albendazole (30 nM) for 24h and cell cycle distribution was then assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation by flow cytometry. The panels at the bottom indicate representative dot-plots showing the percentages of AV- and/or PI-positive cells. Data in the right panels indicate the percentages of cells in the different phases of cell cycle (upper inset) and the percentage of cell death (bottom inset), as assessed by flow cytometry analysis. Values indicate mean ± SD of 4 separate experiments. (Unstimulated vs albendazole-treated cells; *P<0.05). B) Albendazole treatment enhances the expression of CDK2 phosphorylation. HaCaT cells were either left untreated or stimulated with albendazole for 24h. p-CDK2 (Tyr15), CDK2, CDC25A, CDC25B, CDC25C and β-actin expression was analyzed by Western blotting. One of 3
representative experiments in which similar results were obtained is shown. C) Albendazole treatment does not change CDC25A mRNA expression. HaCaT cells were incubated with albendazole for 4, 8 and 24h and CDC25A transcripts were evaluated by real-time PCR. Levels are normalized to β-actin. Values are mean ± SEM of three experiments. Unst: unstimulated.

Figure 6. Albendazole treatment induces PKR-mediated eIF2α phosphorylation and inflammatory molecules inhibition in keratinocytes. A) HaCaT cells were incubated for 5, 15, 30, 60 and 120 minutes with albendazole and p-eIF2α (Ser51), eIF2α, p-PKR (Thr446), PKR and β-actin expression was analyzed by Western blotting. One of 3 representative experiments in which similar results were obtained is shown. Time-course studies show that phosphorylation of PKR precedes phosphorylation of eIF2α. B) HaCaT cells were transfected with either scrambled-siRNA or PKR-siRNA (both used at 100nM). After 24 h, cells were incubated for further 30' in presence or absence of albendazole and p-eIF2α (Ser51), eIF2α, PKR and β-actin were analyzed by Western blotting. One of 3 experiments in which similar results were obtained is shown. C) Albendazole reduces IL-6, IL-8, IL-1β and CCL5 mRNA expression in TNFα-activated HaCaT cells. RNA transcripts were determined by Real-time PCR and normalized to β-actin. Data indicate mean ± SEM of four separate experiments. (Unstimulated vs TNFα-treated cells; *P<0.05). (TNFα-treated vs TNFα+albendazole-treated cells; **P<0.05).

D) Down-regulation of PKR expression with a specific siRNA (100nM) prevented albendazole-mediated inhibitory effect on IL-6, IL-8, IL-1β, and CCL5 expression in TNFα-activated HaCaT cells). RNA transcripts were determined by Real-time PCR and normalized to β-actin. Data indicate mean ± SEM of three separate experiments (PKRsiRNA-treated vs PKRsiRNA+TNFα-treated cells *P<0.05).

Figure 7. Albendazole treatment induces PKR-mediated eIF2α phosphorylation in vivo.
Representative Western blots for p-eIF2α (Ser51), eIF2α, CDC25A, p-PKR (Thr446), PKR and β-actin. Total proteins were extracted from the shaved back skin of mice treated with Aldara and Aldara+albendazole for 4 consecutive days. Albendazole (30μg) was topically applied each day starting 12 hours after Aldara treatment (12 mice/group).
Figure 1

A

Vehicle  Aldara  Aldara+ Albendazole

![Image of mice with PASI score chart showing significant differences.]

B

Vehicle  Aldara  Aldara+ Albendazole

![Image of histological sections showing epidermal thickness.](%20*p%2C%20**p*]

C

IgG  ki67

Vehicle  Aldara  Aldara+ Albendazole

![Image of histological sections showing ki67 expression.](%20*p%2C%20**p*]

D

Aldara  -  -  +  +  Albendazole

![Image of western blot showing K6A, K16, and β-Actin levels.](%20*p%2C%20**p*)
Figure 2

A

IgG

CD3

Ly6G

Covered area of CD3+ cells (%)

Covered area of Ly6G+ cells (%)

Aldara

Aldara+Albendazole

B

IL-6 mRNA relative expression

TNF-α mRNA relative expression

IL-1β mRNA relative expression

IL-17A mRNA relative expression

CCL17 mRNA relative expression

IL-36α mRNA relative expression

IL-36β mRNA relative expression

IL-36γ mRNA relative expression

CXCL1 mRNA relative expression

CXCL2 mRNA relative expression

CXCL5 mRNA relative expression

Vehicle

Aldara

Aldara+Albendazole

* p < 0.05

** p < 0.01
Figure 4

A

B

C

D

Cell death (%)
**Figure 5**

A

![BrdU Area](image)

![AnnV](image)

![Cell death](image)

B

![Albendazole](image)

C

![CDC25A mRNA expression](image)
Figure 6

A

|            | 0' | 5'  | 15' | 30' | 60' | 120' |
|------------|----|-----|-----|-----|-----|------|
| Albendazole|    |     |     |     |     |      |
| 37KDa      |    |     |     |     |     | 1.0  |
| 37KDa      |    |     |     |     |     | 1.5  |
| 74KDa      |    |     |     |     |     | 2.0  |
| 74KDa      |    |     |     |     |     | 2.5  |
| 42KDa      |    |     |     |     |     | 3.0  |

B

|            | PKR siRNA | Albendazole | PKR siRNA | Albendazole |
|------------|-----------|-------------|-----------|-------------|
| 74KDa      |           |             |           |             |
| 37KDa      |           |             |           |             |
| 37KDa      |           |             |           |             |
| 42KDa      |           |             |           |             |

C

- IL-6 mRNA relative expression
  - Unst.
  - Albendazole
  - TNF-α
  - TNF-α+Albendazole
- IL-8 mRNA relative expression
  - Unst.
  - Albendazole
  - TNF-α
  - TNF-α+Albendazole
- IL-1β mRNA relative expression
  - Unst.
  - Albendazole
  - TNF-α
  - TNF-α+Albendazole
- CCL5 mRNA relative expression
  - Unst.
  - Albendazole
  - TNF-α
  - TNF-α+Albendazole

D

- IL-6 mRNA relative expression
  - Unst.
  - CTR siRNA
  - PKR siRNA
  - PKR siRNA+TNF-α
  - PKR siRNA+Albendazole
- IL-8 mRNA relative expression
  - Unst.
  - CTR siRNA
  - PKR siRNA
  - PKR siRNA+TNF-α
  - PKR siRNA+Albendazole
- IL-1β mRNA relative expression
  - Unst.
  - CTR siRNA
  - PKR siRNA
  - PKR siRNA+TNF-α
  - PKR siRNA+Albendazole
- CCL5 mRNA relative expression
  - Unst.
  - CTR siRNA
  - PKR siRNA
  - PKR siRNA+TNF-α
  - PKR siRNA+Albendazole
Figure 7

- 37KDa
- 37KDa
- 67KDa
- 74KDa
- 74KDa
- 42KDa

Aldara

- - + +

Albendazole

| Protein | Description |
|---------|-------------|
| 37KDa   | p-eIF2αSer51 |
| 37KDa   | eIF2α        |
| 67KDa   | CDC25A       |
| 74KDa   | p-PKRThr446  |
| 74KDa   | PKR          |
| 42KDa   | β-Actin      |