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The Retinoid X Receptor Agonist, 9-cis UAB30, Inhibits Cutaneous T-cell Lymphoma Proliferation Through the SKP2-p27kip1 Axis

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Abstract

Background: Bexarotene (Targretin®) is currently the only FDA approved retinoid X receptor (RXR) -selective agonist for the treatment of cutaneous T-cell lymphomas (CTCLs). The main side effects of bexarotene are hypothyroidism and elevation of serum triglycerides (TGs). The novel RXR ligand, 9-cis UAB30 (UAB30) does not elevate serum TGs or induce hypothyroidism in normal subjects.

Objectives: To assess preclinical efficacy and mechanism of action of UAB30 in the treatment of CTCLs and compare its action with bexarotene.

Methods: With patient-derived CTCL cell lines, we evaluated UAB30 function in regulating growth, apoptosis, cell cycle check points, and cell cycle-related markers.

Results: Compared to bexarotene, UAB30 had lower half maximal inhibitory concentration (IC₅₀) values and was more effective in inhibiting the G1 cell cycle checkpoint. Both rexinoids increased the stability of the cell cycle inhibitor, p27kip1 protein, in part, through targeting components involved in the ubiquitination–proteasome system: 1) decreasing SKP2, a F-box protein that binds and targets p27kip1 for degradation by 26S proteasome and 2) suppressing 20S proteasome activity (cell line-dependent) through downregulation of PSMA7, a component of the 20S proteolytic complex in 26S proteasome.

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Conclusions: UAB30 and bexarotene induce both early cell apoptosis and suppress cell proliferation. Inhibition of the G1 to S cell cycle transition by rexinoids is mediated, in part, through downregulation of SKP2 and/or 20S proteasome activity, leading to increased p27kip1 protein stability. Because UAB30 has minimal effect in elevating serum TGs and inducing hypothyroidism, it is potentially a better alternative to bexarotene for the treatment of CTCLs.

Keywords

Cutaneous T-cell lymphoma; 9-cis UAB30; Bexarotene; SKP2; p27kip1; PSMA7

1. Introduction

Cutaneous T-cell lymphomas (CTCLs) are characterized by the localization of neoplastic T lymphocytes in the skin. The most common forms of CTCLs are mycosis fungoides (MF) and Sézary syndrome (SS), which account for 65% of CTCLs [1]. The clinical features of MF are erythematous patches, plaques, and, in severe cases, pruritus and tumor formation [2]. SS is an advanced form of CTCL that includes skin lesions with neoplastic CD4+ T lymphocytes in the blood. In the United States, there are ~1500 new cases reported each year, and the incidence is increasing [3, 4]. The etiology of this disease is not fully understood and, presently, there is no cure.

Because CTCL is an indolent disease, Targretin/Bexarotene, a retinoid X receptor (RXR) ligand (rexinoid) is commonly prescribed long-term to suppress its progression, Fig. 1a [5]. However, the primary side effects of bexarotene are hypothyroidism and elevation of serum triglycerides (TGs) [6, 7]. Hypertriglyceridemia, which can lead to pancreatitis, is a risk factor for cardiovascular disease [8, 9]. Consequently, CTCL patients taking bexarotene are concomitantly prescribed a statin along with tetraiodothyronine as thyroid hormone replacement. This complex regimen increases cost, reduces compliance, and may elicit additional side effects from statins. Thus, identifying low-toxicity drugs that effectively treat CTCL but do not elevate TGs is a high priority among dermatologists.

A potential candidate to replace bexarotene for the treatment of CTCL patients is the rexinoid, 9-cis UAB30 (UAB30), Fig. 1a. Like bexarotene, it binds specifically to RXR α (IC_{50} 284 nM, EC_{50} 118 nM) and not to RAR α , β , or γ [10]. Unlike bexarotene, UAB30 does not increase TGs in rats, dogs, or normal human subjects [11–13] nor does it induce hypothyroidism in the latter (unpublished, as per communication with Dr. Howard H. Bailey). In rat liver, PPAR α /RXR- and LXR/RXR-activated transcripts such as SCD-1 and SREBP-1 (associated with increased TG/lipid biosynthesis) are stimulated by bexarotene, but not by UAB30, suggesting a tissue-specific difference in the interaction of transcriptional regulation between these two rexinoids [14, 15].

UAB30 has shown promising effects in preventing development of breast cancers [16–18], leading to a phase I clinical trial as a chemopreventive agent for breast cancer [13]. Additionally, preclinical studies indicate UAB30's potential in treating pediatric cancers [19, 20]. However, its use as a chemotherapeutic agent for the treatment of CTCLs has not been evaluated. Here, by use of patient-derived CTCL cell lines, we present findings on the

efficacy and the mechanism of action of UAB30 for the potential treatment of CTCL and compare its effects with those of bexarotene.

2. Materials and methods

2.1. Reagents

9-cis-UAB30 [(2E, 4E, 6Z, 8E)-8- (3',4'-dihydro-1'(2'H)-naphthalen-1'-ylidene)-3,7-dimethyl-2,4,6-octatrienoic acid] was synthesized as reported [16]. Bexarotene (LGD1069; 4-[1-(3, 5, 5, 8, 8-pentamethyl-5, 6, 7, 8-tetrahydro-2-naphthyl)ethenyl] benzoic acid) was obtained from Sigma-Aldrich Corporation, St. Luis, MO. Stock concentrations of UAB30 and bexarotene were dissolved in dimethylsulfoxide (DMSO) and stored in dark vials at -80°C . Antibody to p27kip1 (602902, poly6029) was obtained from BioLegend, San Diego, CA. Antibodies to SKP2 (sc-7164, H-435), PSMA7 (sc-100456, GH6) and β -actin (sc-47778, C4) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Antibody to CKS1 (36–6800) was obtained from Invitrogen, Thermo-Fisher Scientific Inc., Waltham, MA.

2.2. Cell lines

The patient-derived CTCL cell line, MyLa 2973 (MyLa), was provided by Dr. R. Dummer, University Hospital of Zurich, Switzerland. MyLa is a clonal cell line originally derived from a single plaque of an 82-year-old Caucasian male diagnosed with MF [21]. The HuT 78 (ATCC[®] TIB-161), HH (ATCC[®] CRL-2105) and Jurkat, clone E6–1 (ATCC[®] TIB-152) cell lines were obtained from ATCC, Rockville, MD. HuT 78 is derived from peripheral blood of a 53-year-old Caucasian male diagnosed with SS. HH is derived from peripheral blood of a 61-year-old Caucasian male diagnosed with CTCL. Jurkat is acute T cell leukemia cells established from peripheral blood of a 14-year-old boy. They were maintained in RPMI 1640 media (Thermo-Fisher Scientific Inc.), supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Atlanta Biological Inc., Flowery Branch, GA), and 0.5% penicillin-streptomycin in a humidified atmosphere at 37°C with 5% CO_2 . All lines were used within ten passages from the obtained date. All cell lines were determined to be free of mycoplasma [22].

2.3. Cell viability assays and determination of half maximal inhibitory concentration (IC_{50}) values for UAB30 and bexarotene

In figure 1b, cells ($2 \times 10^4/\text{ml}$ with 10 ml of cells in 100 mm dish, triplicate dishes/treatment) were treated with various concentrations of UAB30 or bexarotene or with dimethylsulfoxide (DMSO) as solvent control for 24 or 48 h. Harvested cells were centrifuged and suspended in media. Live cells (not stained with trypan blue) were counted by hemocytometer. On determination of IC_{50} values for UAB30 and bexarotene, cells were seeded at $1 \times 10^4/\text{ml}$ as 1 ml/well in 24 well plates and treated with various concentrations of rexinoids or DMSO in quadruplicate wells/treatment for 48 h.

2.4. Flow cytometry assay of cell apoptosis

Apoptosis was analyzed by using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to manufacturer's protocol. HuT 78, MyLa and Jurkat cells (1×10^6

cells/100 mm dish) were treated with rexinoids or DMSO in triplicate dishes/treatment for 24h or 48h, see legend. Cells were collected, washed twice with cold PBS, centrifuged at 1500 rpm for 5 min, and resuspended in binding buffer. Cells were transferred to a 5 ml culture tube and annexin V-FITC and propidium iodide (PI) staining solution were added. Additionally, a set of DMSO-treated cells were either not stained, stained with PI only or stained with annexin V only and used for compensation. Cells were gently mixed, and incubated for 15 min at room temperature in the dark. After that, 400 μ l of 1 $\times$ binding buffer were added to each tube and samples were analyzed by LSRII flow cytometry (BD Biosciences). Data are represented as % cell population not stained with Annexin V or PI representing live cells, stained with Annexin V⁺ PI⁻ stained cells representing early apoptotic cells, stained with Annexin V⁺ PI⁺ cells representing late apoptotic cells and stained with PI only representing necrotic cells, Tables 1, 2 and 3.

2.5. Cell cycle analyses

CTCL cells (5×10^4 /ml) were treated with UAB30, bexarotene, or DMSO for 24 or 48 h in triplicate dishes. Cells were harvested at designated time points, rinsed, and fixed with 100% ethanol. DNA analyses were performed according to a modified protocol [23, 24]. Briefly, cells were treated with 200 μ g/ml of RNase, stained with 20 μ g/ml of propidium iodide and analyzed by flow cytometry (Becton Dickinson FACS Calibur, BD Biosciences, San Jose, CA). DNA contents in various stages of the cell cycle were assessed by ModFitLT V3.3.11 (Mac).

2.6. Western blot analyses

CTCL cells, plated as above, were incubated with UAB30 or bexarotene or with DMSO for 24 h. Cells were lysed with RIPA buffer containing protease inhibitors (Roche, Indianapolis, IN), centrifuged, and the protein concentrations in the supernatants were determined by the bicinconinic acid (BCA) method (Thermo Fisher Scientific Inc.). Equal amounts of protein lysates were separated by 8% or 10% SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membranes, and probed with primary antibodies followed by the appropriate secondary antibodies coupled to horseradish peroxidase.[25] Protein bands were visualized with chemiluminescence substrate (Amersham ECL, GE Healthcare, Pittsburgh, PA) and exposed to blue X-ray films. Densitometric analyses of the protein bands were performed with Image Software.

2.7. Real-time polymerase chain reaction (qPCR)

Total RNA was extracted by TRIzol® reagent (Life Technology). For qPCR analysis, 2 μ g of total RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen, Thermo-Fisher Scientific) and random hexamers. Amplification of SKP2, p27kip1, CKS1B, and β -actin mRNA (Supplement, Table S1 for primer sets) was performed using PowerUp SYBR Green PCR Master Mix according to the manufacturer's specifications (Applied Biosystems Incorporated, Foster City, CA) and analyzed with a Roche LC480 Light Cycler system (Roche, Mannheim, Germany). Relative expressions of the target mRNA normalized with β -actin mRNA were determined by the $-C_p$ method, as recommended by the manufacturer.

2.8. SKP2 mRNA decay

MyLa and HuT 78 cells cultured at same cell density for 16 h were treated with actinomycin D at 10 µg/ml or 0.5 µg/ml, respectively, followed by UAB30, bexarotene, or DMSO. Preliminary studies were performed to select non-toxic optimum concentration of actinomycin D for each cell line. Additionally, at chosen concentration of actinomycin D, they were tested to ensure that it still inhibits transcription. After drug treatment, total RNAs were isolated at various time points, and qRT-PCR analyses were performed as described above. SKP2 mRNA expression levels were normalized with β-actin mRNA for MyLa cells or 18S rRNA for HuT 78 cells. To obtain the half-life ($t_{1/2}$) of SKP2 mRNA, the best fit linear curve was drawn on a log-linear plot of the SKP2 mRNA/β-actin mRNA (MyLa) or SKP2mRNA/18S rRNA (HuT 78) ratio (labeled as SKP2 mRNA remaining) versus time (h) post actinomycin D treatment. $T_{1/2}$ was extrapolated from 50% of SKP2 mRNA remaining. Both internal controls (β-actin mRNA and 18S rRNA) were not affected by retinoids, data not shown.

2.9. 20S Proteasome activity assay

Proteasome activity was determined by use of a 20S proteasome activity assay kit (Millipore, Billerica, MA) based on detection of the fluorophore, 7-amino-4 methylcoumarin (AMC) after cleavage of the labeled substrate, LLVY-AMC. HuT 78 and MyLa cells were treated with retinoids for 12–24 or 18–48 h. HH cells were treated with retinoids for 24 h. Harvested cells were lysed in 25 mM Hepes, pH 7.5, with 0.5% NP-40 on ice, centrifuged, and equal volumes of supernatants were incubated with LLVY-AMC to determine the 20S proteasome enzymatic activity. The fluorescence intensity of the product was monitored with Tecan, Infinite 200 PRO, Tecan Group Ltd, Männedorf, Switzerland. The protein concentrations of cell lysates were determined by the BCA method and used to normalize the final data expressed as nM/µg.

2.10. Statistics

Data are reported as means ± standard deviation (SD), unless stated otherwise. Paired t-tests were used for comparison between groups. P values < 0.05 were statistically significant. All analyses were performed with SigmaPlot 11.0. All data are representative of two to three additional independent experiments.

3. Results

3.1. UAB30 suppresses cell viability of CTCL cell lines as well as bexarotene

Analyses of CTCL cell lines representative of MF (MyLa) and SS (HuT 78) indicate that UAB30 is as effective as bexarotene in suppressing cell viability. In MyLa cells, treatment with UAB30 at 50 µM for 24 h significantly decreased cell numbers relative to control cells. By 48 h of treatment with either retinoid at 25 or 50 µM, cell viability was inhibited relative to control cells (Fig. 1b, top panels).

In contrast to MyLa cells, HuT 78 cells were more sensitive to UAB30 and bexarotene. Both retinoids at concentrations of 5, 10, or 25 µM suppressed cell viability by 24 h (Fig. 1b, lower left panel), which was not observed in MyLa cells when treated with 5 or 10 µM

concentrations (data not shown). By 48 h, UAB30 and bexarotene at 25 μ M caused decrease in numbers of HuT 78 cells relative to control cells. Furthermore, UAB30 significantly suppressed cell viability compared to bexarotene-treated cells (Fig. 1b, lower right panel).

3.2. The half maximal inhibitory concentration (IC₅₀) values of UAB30 and bexarotene for CTCL cell lines after 48 h of treatment

To determine the IC₅₀ values, MyLa, HuT78, and HH (an aggressive form of CTCL) cells were treated with various concentrations of retinoids for 48 h. The IC₅₀ values of UAB30 for MyLa, HuT 78, and HH cells were 34.7, 5.1, and 22.4 μ M respectively, whereas the IC₅₀ values of bexarotene were 39.8, 24.5, and 23.6 μ M respectively (Fig. 1c). Both HuT 78 and HH cells are more sensitive to retinoids relative to MyLa cells. Additionally, for HuT 78 cells, UAB30 is more effective in increasing cell death.

3.3. UAB30 and bexarotene induce early cell apoptosis

Data from cell viability and IC₅₀ studies prompted us to examine the effect of UAB30 on inducing cell apoptosis and/or suppressing cell proliferation. To first determine the effect of UAB30 on early cell apoptosis, annexin V assays were performed. MyLa and HuT 78 cells were treated with 25 μ M of UAB30 and bexarotene for 48 or 24 h, respectively. The rationale in treating HuT 78 cells with retinoids for 24 h is based on their higher sensitivity to the drugs as noted in the IC₅₀ experiments (Fig. 1c). In MyLa cells, after 48 h of treatment with UAB30 or bexarotene, a significant increase in early cell apoptosis was observed when compared to DMSO control cells. The average percent cell population stained with annexin V only (indicative of early apoptosis) for control, UAB30 and bexarotene-treated cells were 1.6 ± 0.5 , $7.5 \pm 0.7^{**}$, and $9.4 \pm 1.1^*$, respectively (mean $\pm$ standard deviation; *, $p < 0.001$, **, $p < 0.0001$ compared to control; $n=3$) (Table 1 and Fig. 1d, top panels, Q4 representing cell stained with annexin V only).

In HuT 78 cells, 24 h treatment with retinoids showed a significant increase in early apoptosis in UAB30-treated cells compared to control. The average percent cell population stained with annexin V only for control, UAB30 and bexarotene-treated cells were 2.2 ± 1.0 , $5.4 \pm 1.3^{**}$, and 3.3 ± 1.2 , respectively (**, $p < 0.0001$ compared to control; $n=6$) (Table 2 and Fig. 1d, lower panels).

3.4. UAB30 and bexarotene may also be effective in suppressing cell proliferation and/or inducing apoptosis of Jurkat cells.

To determine whether the observed effects on CTCL cell lines shown above by UAB30 and bexarotene might also affect non-CTCL cells, we determined the cell viability, early apoptosis, and their IC₅₀ values in an acute T-cell leukemia cell line, Jurkat. Treatment with retinoids at 25 and 50 μ M for 24 h showed significant decrease in cell number compared to DMSO control (Fig. 2a). Since the total number of cells seeded/well was thirty thousand, it appears that at 25 μ M, UAB30 likely suppressed Jurkat cell proliferation and its effect is better than bexarotene ($p < 0.05$) and that only at much higher concentrations (i.e. 50 μ M) are cell apoptosis induced by retinoids. This is further supported by our annexin V analyses indicating that cells treated with 25 μ M of UAB30 and bexarotene for 24 h did not show

significant early apoptosis when compared with control cells (1.6 ± 0.5 , 1.3 ± 0.3 and 1.1 ± 0.2 , respectively) (Fig. 2b and Table 3).

In Jurkat cells, the IC_{50} values for UAB30 and bexarotene after 48 h of treatment were 32 and 46 μM , respectively (Fig. 2c). Comparison of the IC_{50} graphs to those of CTCL cells indicated that Jurkat cells are much more resistance to cell apoptosis when treated with bexarotene, while the effects of UAB30 are comparable with MyLa cells. Since the main focus of our study is on CTCL, additional studies were performed to further define the mechanism by which UAB30 and bexarotene suppress cell proliferation in CTCL cell lines.

3.5. UAB30 is as effective as or better than bexarotene in blocking at the G1 cell cycle checkpoint in CTCL cell lines

In addition to rexinoids inducing early apoptosis in both MyLa and HuT 78 cells, there were also substantial live cells present after rexinoid treatment. In MyLa cells, after 48h of treatment with UAB30 and bexarotene the percent live cells were 76 ± 1.1 and 64 ± 3.8 , respectively (Table 1). In HuT 78 cells, after 24 h of treatment with UAB30 and bexarotene the percent live cells were 87 ± 3.8 and 86 ± 4.5 , respectively (Table 2). To determine the possibility of UAB30 and bexarotene in suppressing cell proliferation, we assessed their effect on various stages of cell cycle. MyLa cells were treated with rexinoids at 25 μM for 24 or 48 h, and cell cycle analyses were performed by flow cytometry. After 24 h of treatment, the percent cell population at different stages showed no appreciable effect of rexinoids (data not shown). However, by 48 h, both rexinoids increased the percent cell population at G0/G1 and concomitantly decreased percent cell population in the S stage of the cell cycle relative to DMSO-treated cells (Fig. 3a). The percent cell population at the S stage of UAB30-treated cells was lower ($p = 0.002$) than that for bexarotene. Only UAB30 increased the percent cell population at G2/M stage of cell cycle relative to DMSO-treated cells, indicating that it may inhibit at the G2/M checkpoint. Analyses of ratios G1/S and G2/G1 from percent cell population at various cell cycle stages confirmed that both rexinoids inhibits G1 to S cell cycle transition and UAB30 exhibited a ($p < 0.05$) greater effect than bexarotene (Table 4) in arresting cell cycle at checkpoint G1, with an average fold increase of 1.94 ± 0.26 (UAB30) vs 1.35 ± 0.12 (bexarotene) relative to DMSO. In contrast, G2/G1 ratio analysis indicated that both rexinoids have no effect significant effect in arresting cell cycle at G2/M checkpoint.

Since we have shown that HuT 78 cells are more sensitive to rexinoids (Fig. 1b and 1c), these cells were also treated with lower concentrations (5 and 10 μM) and for a shorter time period (24 h). Both rexinoids at 5 μM (data not shown) and higher were effective in arresting at the cell cycle G0/G1 (Fig. 3b). UAB30 at 10 and 25 μM increased ($p < 0.05$) the percent cell population at the G0/G1 stage over that of bexarotene-treated HuT 78 cells. Additionally, UAB30 at 25 μM decreased ($p < 0.001$) the percent cell population at the S stage of cell cycle compared to bexarotene-treated cells (Fig. 3b).

3.6. Inhibition of cell cycle at G1 checkpoint by rexinoids is regulated, in part, through an increase in the cyclin-dependent kinase inhibitor, p27kip1, and a decrease in SKP2

Inhibition of the cell cycle at G1 checkpoint suggests that the rexinoids up-regulate the level of cyclin-dependent kinase inhibitor, p27kip1, a regulator in arresting cell proliferation. In various types of cancer cells, p27kip1 protein levels are markedly suppressed [26–28]. In MyLa cells, both rexinoids significantly increased its levels after 24 h of treatment (Fig. 4a).

The increases of p27kip1 protein suggest that the rexinoids upregulate its transcript and/or increase its stability. Since p27kip1 is largely regulated at the post-transcriptional level [29], we first investigated the mechanism by which UAB30 and bexarotene increased its stability. Various molecular pathways have been postulated to regulate the stability of p27kip1 protein [30–34]. One pathway involves regulation by the S-phase kinase-associated protein 2 (SKP2), an F-box protein in the Skp, cullin, F-box containing (SCF) E3 ubiquitin ligase complex that binds phosphorylated p27kip1, which is subsequently degraded by 26S proteasomes [31, 32, 35]. Because SKP2 is also overexpressed in cancer cells and is a major driver of the G1 to S transition [36], we determined whether rexinoid inhibition of the G1 checkpoint is mediated through suppression of SKP2, thereby increasing the stability of p27kip1 protein. Indeed, we found in MyLa cells that UAB30 at 10 and 25 μ M and bexarotene at 10 μ M suppressed SKP2 after 24 h of treatment (Fig. 4a).

With respect to HuT 78 cells where rexinoids inhibited the G1 cell cycle checkpoint by 24 h, we observed a consistent time-dependent increase of p27kip1 protein by as early as 13 h, which persisted at 24 h when treated with both rexinoids, although the absolute levels of p27kip1 appeared to decrease by 24 h. Both rexinoids suppressed SKP2 in a time-dependent manner from 0 to 24 h. By 13 h of treatment, UAB30 at 10 μ M and bexarotene at 10 or 25 μ M suppressed the level of SKP2 compared to control cells, and this suppression largely persisted to 24 h of treatment (Fig. 4b).

In HH cells, both rexinoids stimulated an increase in p27kip1 protein with a concomitant decrease of SKP2 protein expression, (Fig. 4c).

3.7. UAB30 and bexarotene do not suppress the transcript of p27kip1 or CKS1B, but decrease the stability of the SKP2 transcript

To assess the possibility that rexinoids upregulate p27kip1 at the transcript level in addition to lowering SKP2, the steady-state level of its mRNA was measured. UAB30 and bexarotene did not elevate the mRNA of p27kip1 in MyLa, HuT 78, or HH cells (Fig. 5a). These findings suggest that the increase of p27kip1 protein observed in the presence of the rexinoids is likely due to attenuation of its degradation by 26S proteasome mediated through rexinoids decreasing SKP2.

To determine if the lowering of SKP2 protein by rexinoids is regulated at the transcriptional level, qPCR analyses were performed. In all CTCL cell lines, treatment with rexinoids for 24 h suppressed the steady-state level of SKP2 mRNA compared to control cells (Fig. 5b). A decrease in the steady-state level of SKP2 mRNA by rexinoids suggests that they may directly suppress transcription or decrease the stability of SKP2 mRNA. To test this, we inhibited transcription by treating the cells with the transcriptional inhibitor, actinomycin D,

followed by the addition of rexinoids and DMSO. Determination of half-life ($t_{1/2}$) of SKP2 mRNA in CTCL cell lines (MyLa and HuT 78) indicated that both rexinoids accelerated the degradation of SKP2 mRNA relative to DMSO-treated cells. In MyLa cells, $t_{1/2}$ of SKP2 mRNA is significantly lower in UAB30 or bexarotene-treated cells (14 ± 7 and 10 ± 3 h, respectively) when compared with control cells (51 ± 14 h) (Fig. 5c, top panel). Likewise, in HuT 78 cells, $t_{1/2}$ of SKP2 mRNA is significantly lower in cells treated with UAB30 or bexarotene (7.4 ± 1.6 and 8.5 ± 0.3 , respectively) when compared with control cells (31.2 ± 5.5) (Fig. 5c, lower panel). These findings suggest the possibility of rexinoids functioning through the RXR-independent pathway in regulating SKP2 mRNA degradation.

In addition to SKP2 as a regulator of the stability of the p27kip1 protein, CKS1B, an adaptor protein that binds to SKP2, contributes to increasing the degradation of p27kip1 protein [37]. Therefore, we determined if the increased stability of the p27kip1 protein was accomplished by rexinoids decreasing the transcript of CKS1B. Analysis of steady-state level of CKS1B mRNA by qPCR indicated that UAB30 and bexarotene did not suppress the expression of its transcript in MyLa, HuT 78, and HH cells (Fig. 5d), nor did they suppress its protein expression (Supplement Fig. S1, shown for MyLa and HuT 78 cells).

3.8. Rexinoids attenuate PSMA7 protein expression , a component of the 20S catalytic subunit of the 26S proteasome complex in both CTCL cell lines, but the 20S proteasome activity was suppressed by both rexinoids only in HuT 78 cells and by UAB30 in HH cells

In addition to suppression of SKP2 that increased stability of the p27kip1 protein, a decrease in the activity of 26S proteasome may also increase the amount of p27kip1. Preliminary proteomic data (Supplement, Fig. S2) for MyLa cells treated with UAB30 or bexarotene indicated that UAB30 significantly attenuated PSMA7, proteasome subunit alpha type-7 of the 20S catalytic subunit of the 26S proteasome complex. The 26S proteasome complex comprises two 19S regulatory cap subunits and the 20S catalytic subunit involved in degrading ubiquitinated proteins [38]. A decrease in PSMA7 could compromise the assembly of 20S and its proteolytic activity, and consequently increase the accumulation of p27kip1.

To validate our proteomic finding on PSMA7, Western blot and qRT-PCR analyses were performed for MyLa, HuT 78, and HH cells. PSMA7 protein was suppressed in CTCL cells by UAB30 at 25 μ M and as well as bexarotene at 10 and 25 μ M after 24 h of treatment (Fig. 6a). Rexinoids, however, did not regulate the expression of PSMA7 at the transcriptional level in all cell lines, since treatment with 25 μ M rexinoids did not lower the steady-state level of PSMA7 mRNA (Fig. 6b).

To determine if suppression of PSMA7 by rexinoids affected 20S proteasome activity, MyLa and HuT 78 cells were treated with 25 μ M UAB30 or bexarotene for 18–24 h and 12–24 h, respectively. 20S proteasome activity was not suppressed in MyLa cells, but was suppressed in HuT 78 cells at 18 h by UAB30 and at 24 h by both UAB30 and bexarotene (Fig. 6c). In HH cells treated with rexinoids for 24 h only, UAB30 significantly suppressed 20S proteasome activity, while bexarotene did not.

4. Discussion

UAB30 is as effective as bexarotene suppressing cell viability and inducing early cell apoptosis of CTCL cell lines. The IC_{50} values for UAB30 in inhibiting cell survival of CTCL cells were lower than those for bexarotene. Additionally, UAB30 in MyLa and HuT 78 cells is as effective as or better than bexarotene in suppressing cell proliferation through arresting the cell cycle at the G1 checkpoint. The arrest at the cell cycle G1 checkpoint by UAB30 and bexarotene is, in part, regulated through decreasing SKP2 mRNA in CTCL cells and also in decreasing 20S proteasome activity in HuT 78 cells and HH cells (by UAB30), thereby increasing stability of the p27kip1 protein, which inhibits cell cycle progression.

The effects of UAB30 and bexarotene are not specific to CTCL cells only and therefore, they can be applicable for the treatment of other types of T-cell disorders. Using the acute T-cell leukemic Jurkat cells, we showed that UAB30 is a more effective chemotherapeutic agent than bexarotene as indicated by its significant decrease in cell viability at 25 μ M and also lower IC_{50} value.

Comparison of MF (MyLa) and SS-derived (HuT 78) and HH, an aggressive form of CTCL, cells lines indicated differential sensitivities to retinoids. HuT 78 and HH cells are more sensitive than MyLa cells to retinoids, as is evident by the lower IC_{50} values and in HuT78 cells by the early G1 cell cycle arrest at 24 instead of 48 h. Although MF is generally considered as a milder form of CTCL, the fact that the MyLa cell line is more resistant to retinoid treatment is probably because 1) it was derived from a patient manifesting the later/malignant stage of MF, and/or 2) in cultures, it has transformed into a more aggressive line. Nevertheless, UAB30 is more effective in arresting cell proliferation at the G1 checkpoint, with a lower IC_{50} value than that of Bexarotene.

Both UAB30 and bexarotene decrease the stability of the SKP2 transcript, which correlates with the attenuation of its protein and subsequent increase in p27kip1 protein in CTCL cells. SKP2 belongs to the family F-box proteins, which interact with CKS1 and SKP1. These proteins form complexes with other components (Cullin 1, ROC1 and UBC3) involved in protein ubiquitination (Fig. 7). SKP2 binds to phosphorylated (T187) p27kip1, resulting in its ubiquitination and subsequent degradation by 26S proteasome [39]. We postulate that, in CTCL cells, p27kip1 is the primary tumor suppressor and inhibitor of cell proliferation. Mice that lack the *p27kip1* gene (*Cdkn1b*) are increased in size, with enlarged organs [40], in contrast to mice that lack the *p21* gene (*Cdkn1a*), also a cyclin-dependent kinase inhibitor [41]. Our observed inverse relationship between SKP2 and p27kip1 in control CTCL cells is consistent with other studies [26, 27, 42, 43]. SKP2 overexpression, found in human cancer progression and metastasis, acts as a facilitator to proto-oncogene [43]. Conversely, *Skp2*-deficient mice have smaller organs and slower growth, with accumulation of p27kip1 protein [42]. Overexpression of SKP2 in T cells in mice harboring activated N-*ras* oncogene leads to lymphomagenesis [43]. Thus, downregulation of SKP2 is an important target for the treatment of CTCL.

In contrast to RXR-selective ligands, UAB30 and bexarotene, RAR-ligands (all-trans retinoic acid and 13-cis retinoic acid) and a ligand that interacts with both RXR and RAR

(9-cis retinoic acid) did not show consistent inverse regulation of SKP2 and p27kip1. In some studies enhanced stability of the p27kip1 protein correlated with a decrease in SKP2 protein stability [44–47], downregulation of SKP2 mRNA [48], or no change in the level of SKP2 mRNA or protein [49, 50]. These differential response of SKP2 could be attributed to the various cell types and to their interaction with RAR receptor. With regard to RXR-selective ligands, it has been reported that UAB30, bexarotene, AGN4204, and AGN194204 upregulated the p27kip1 protein, but SKP2 expression was not analyzed [51–53]. Thus, to our knowledge, this is the first to show that in CTCL cells, UAB30 as well as bexarotene suppress G1 cell cycle checkpoint by accelerating the degradation of SKP2 transcript and consequently, its protein with a concomitant upregulation of p27kip1 protein.

In addition to targeting SKP2, we found in HuT 78 cells that the increase in the stability of the p27kip1 protein could also be attributed through rexinoids decreasing PSMA7 protein and consequently, compromising the catalytic activity of 20S proteasome in degrading p27kip1. The inability of rexinoids to suppress 20S proteasome activity in MyLa cells, despite a decrease in PSMA7 protein, is likely due to a compensatory effect in which another subunit takes the place of PSMA7 to maintain an active 20S catalytic proteasome [54]. In HH cells, both rexinoids decreased PSMA7 protein expression, but at 24 h of treatment only UAB30 and not bexarotene suppressed 20S proteasome activity. It is possible that with additional time course study, bexarotene may also suppress the 20S proteasome activity.

Besides the ability of rexinoids suppressing SKP2 and 20S proteasome activity that leads to elevating p27kip1 protein, another possibility is that they may exert a direct effect in stimulating p27kip1 protein synthesis in CTCL cell lines. This is a possibility, because, in a breast cancer cell line, UAB30 has been postulated to stimulate translation initiation of p27kip1 [51].

UAB30, like bexarotene, binds specifically to RXR α (IC_{50} 284 nM) and not to RAR α , β , or γ . Transcriptional activation assays in CV-1 cells indicate that it transactivates through homodimers of RXR α (EC_{50} of 118 nM) [10]. Additional *in vitro* studies indicate that the effective dose of UAB30 in suppressing cell transformation in RK3E cells, cell proliferation, and invasion/migration and in inducing apoptosis is at 1 μ M or higher [18–20, 55–58]. This suggests that UAB30 and also bexarotene at concentration higher than 1 μ M could be acting through an RXR-independent pathway, since we found that both UAB30 and bexarotene at 25 μ M accelerate the degradation of SKP2 mRNA and decrease PSMA7 protein levels without affecting the levels of its mRNA. The possibility of RXR-independent regulation by UAB30 has also been reported, as it inhibits Src activation by docking to its ATP-binding pocket [58]. Additional studies are necessary to elucidate the RXR-independent pathway of action by UAB30 and bexarotene.

The present findings suggest that UAB30 has the potential of moving towards translational studies, because it is as effective as bexarotene in inducing early cell apoptosis and suppressing cell cycle progression of both CTCL cell lines. However, whether the doses (5–25 μ M) used in this study are likely to be achievable *in vivo* needs to be considered. Currently, the phase 1 clinical trial for breast cancer prevention is testing an oral dose of

UAB30 at 240 mg/m²/day in healthy volunteers. Based on previous reported pharmacokinetic data, an oral intake of 20 mg/m²/day of UAB30 resulted in maximum plasma level (C_{max}) of approximately 80 ng/ml [13]. Extrapolation of an oral dose of UAB30 at 240 mg/m²/day will result in approximately 3.3 μM in the plasma (molecular weight is 294.4 g/mole). We believe that an oral intake of UAB30 at 240mg/m²/day if taken with food, the plasma circulation could be higher than 3.3 μM, because in the phase 1 clinical study [13], UAB30 was taken in an empty stomach. Because UAB30 is a hydrophobic compound, the bioavailability of UAB30 when taken with food is likely to increase and consequently, its C_{max} would be higher and possibly, closer to 10 μM. At this dose, UAB30 suppresses SKP2 protein and increases p27kip1 protein in CTCL cells. Therefore, we believe that the *in vitro* tested dose of at least 10 μM is potentially achievable *in vivo*.

In summary, UAB30 is effective in suppressing the progression of CTCL cell lines at the G1 cell cycle checkpoint, and this is mediated, in part, through suppression of SKP2, resulting in elevating p27kip1 protein. Further, UAB30 inhibits 20S proteasome catalytic activity, which could, in HuT 78 and HH cells, contribute to an increase in the level of p27kip1 protein. Although bexarotene has similar mechanistic effects in CTCL cell lines, however, in contrast to UAB30, it elevates serum TGs and induces hypothyroidism. Therefore, UAB30 is potentially a better alternative to bexarotene for the long-term treatment of CTCL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(a)

(b)

(c)

(d)

MyLa_48 h

HuT 78_24 h

Fig. 1.

Structure of 9-cis UAB30 and bexarotene and their effect on CTCL cell viability, apoptosis, and their IC_{50} values. (a) Structure of UAB30 and bexarotene/Targretin. (b) MyLa cells (2×10^4 /ml) were treated with 25 or 50 μ M of UAB30 or Targretin, or with the DMSO solvent control (C). Viable cells were counted after 24 or 48 h of treatment and reported as mean $\pm$ standard deviation (SD), $n=3$ /treatment. Likewise, HuT 78 cells were treated with either rexinoids at 5, 10, or 25 μ M for 24 h or at 25 μ M for 48 h. *, $p<0.05$ and **, $p<0.001$ vs control cells. #, $p<0.05$ vs UAB30-treated cells. (c) MyLa, HuT 78, HH cells (1×10^4 /ml) were treated with increasing concentrations of UAB30 or Targretin for 48 h. Each point corresponds to the average % of live cells relative to control cells, $n=4$ /drug concentration, error bars (SD). IC_{50} values for the rexinoids were extrapolated from semi-log graphs. (d) MyLa or HuT 78 cells ($\sim 1 \times 10^6$ cells) were treated with 25 μ M for 48 and 24 h, respectively, and annexin V analyses were performed. Data were presented as dot plot of propidium iodide (PI) versus Annexin V (V). Each plot is representative of % cell population from a single sample of MyLa (top panels), $n=3$ /treatment or HuT 78 cells (bottom panels), $n=6$ /treatment, treated with DMSO, UAB30 or bexarotene not stained with V or PI in Q3 (live

cells), stained with V^+PI^- in Q4 (early apoptotic cells), stained with V^+PI^+ in Q2 (dead cells) and stained with V^-PI^+ in Q1 (necrotic cells).

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Fig. 2. Effect of UAB30 and bexarotene on Jurkat cell viability, apoptosis, and their IC₅₀ values. (a) Cells were treated with 25 or 50 μM of UAB30 or Targretin for 24 h, mean ± SD, n=3/treatment. **, p < 0.001; #, p < 0.05 (b) Annexin V analysis. Dot plot, see Fig. 1 legend for detail procedure. (c) IC₅₀ values, n=4.

(a) MyLa

(b) HuT 78

Fig. 3.

Rexinoids arrest MyLa and HuT 78 cells at the G1 cell cycle checkpoint. (a) Top panel, MyLa cells, % cell population at different stages of the cell cycle after 48 h of treatment with 25 μM of UAB30 (U) or Bexarotene (T), or with DMSO (n=3/treatment). Lower panels, cell cycle data analyzed with ModFit LT V3.3.11. Results represent one of the triplicate samples in top panel. (b) Top panel, HuT 78 cells, similar as above except cells were treated for 24 h with 10 or 25 μM of rexinoids or DMSO. Lower panels, cell cycle data analyzed as describe above. Data are representative of two to three independent experiments.

(a) MyLa **(b) HuT 78**

(c) HH **Fig. 4.**

UAB30 and bexarotene downregulate SKP2 and concomitantly, increase p27kip1 protein in CTCL cell lines. (a) MyLa cells treated with DMSO (control), UAB30 (UAB), or bexarotene (T) at 10 or 25 μ M for 24 h. Top panels are representative of one Western blot analysis for p27kip1 and SKP2 proteins. Values below the protein bands are ratios of band intensity of p27kip1 or SKP2 normalized to β -actin. Histograms of average values $\pm$ SD of p27kip1 and SKP2 relative to control from multiple Western blot assays. DMSO, n=5–6; UAB 10 and T 25, n=3; UAB 25 and T 25, n=6. (b) Western blot data of p27kip1 and SKP2 for HuT 78 cells treated with rexinoids for 6, 13, or 24 h. For histograms of p27kip1 and SKP2, at 6 h, n=2/treatment, at 13 h, p27kip1, n=5 and SKP2, n=4; at 24 h, p27kip1, n=3 and SKP2, n=6. (c) Western blot and histograms as described above of p27kip1 and SKP2 for HH cells treated with rexinoids for 24 h, n=3/treatment. *, $p < 0.05$ and **, $p < 0.001$ vs control cells.

Fig. 5.

In CTCL cells, retinoids suppress the steady-state levels of SKP2 mRNA, through accelerating transcript degradation, but do not regulate 27kip1 and CKS1B mRNA expression. (a), (b), and (d) MyLa, HuT 78, and HH cells were treated with UAB30 (UAB) or bexarotene (T) at 25 μ M for 24 h and analyzed by qPCR for p27kip1, SKP2, and CKS1, respectively. Histograms show average values of Skp1, p27kip1, and CKS1 mRNA normalized with β -actin mRNA relative to control. p27kip1 mRNA (MyLa cells: n=8–9; HuT 78 cells: n=5–6; HH cells: n=3). SKP2 mRNA (MyLa cells: n=4–5; HuT cells: n=5–7;

HH cells; n=3). CKS1 mRNA (MyLa cells: n=10–11; HuT 78 cells: n=5; HH cells: n=3). *, $p<0.05$ vs control cells. (c) MyLa cells (top panel) were treated with actinomycin D (10 $\mu\text{g/ml}$) followed by DMSO or 25 μM of UAB30 or bexarotene/Targretin. Total RNAs were isolated at various time points and analyzed by for SKP2 mRNA by qPCR, n=3–4/treatment/time point. SKP2 mRNA expression levels were normalized to β -actin mRNA. The half-life of SKP2 mRNA was calculated by drawing the best fit linear curve on a log-linear plot of the SKP2/ β -actin ratio versus time (h) post-actinomycin D treatment (tx). The time at 50% of SKP2 mRNA remaining is designated the half-life ($t_{1/2}$). Note, the regression line for UAB30 overlaps with that of bexarotene. Likewise, HuT 78 cells (lower panel) were treated similarly with 0.5 $\mu\text{g/ml}$ of actinomycin D. SKP2 mRNA levels were normalized with 18S rRNA, n=3/treatment/time point. *, $p<0.05$; **, $p<0.01$ vs control cells.

(a)

(b)

Fig. 6.

In CTCL cells, retinoids suppress PSMA7 protein, but not its transcript and inhibit 20S proteasome activity in selective cell lines. (a) MyLa, HuT 78 and HH cells were treated with retinoids as in Fig. 4 for 24 h. Top panels, representative of single Western blot analysis of PSMA7 and β -actin. Values below the protein bands are ratios of band intensity of PSMA7 normalized to β -actin. Bottom panels, shows histograms of average fold expression of PSMA7 $\pm$ SD relative to control cells from Western blot assays. HuT 78 cells, n=3/treatment. MyLa cells, control, n=6; UAB 10 and T 10, n=3; UAB 25 and T 25, n=6. HH cells, n=3. (b) qPCR analysis of steady-state PSMA7 mRNA in CTCL cells treated with UAB30 (UAB) or bexarotene (T), as in Fig. 4. MyLa cells, values are mean $\pm$ standard error of mean (SEM) from four independent experiments. HuT 78 cells, mean $\pm$ SD, n=6/treatment. HH cells, mean $\pm$ SD, n=3/treatment. (c) 20S proteasome activity of MyLa, HuT

78, and HH cells, respectively, after treatment with 25 μ M of UAB30 (UAB) or bexarotene (T) at designated times, n=3/treatment. *, p<0.05 and **, p<0.001 vs control cells.

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Fig. 7.

Hypothetical model of the mechanism of action of UAB30 and bexarotene in attenuating CTCL cell proliferation. In CTCL cells (MyLa, HuT 78 and HH), the levels of p27kip1 protein are low and are, in part, suppressed by high levels of SKP2, which targets phosphorylated p27kip1 for ubiquitination and subsequent degradation by 26S proteasome. Treatment with UAB30 or bexarotene suppresses the production of SKP2, resulting in increased p27kip1 protein in CTCL cells. In HuT 78 cells, the increase of p27kip1 protein is also due to the decrease of 20S proteolytic activity of 26S proteasome, which is mediated through the retinoids suppressing the synthesis of PSMA7 protein (a component of the 20S proteolytic complex). In HH cells, UAB30 also decreased 20S proteolytic activity. Accumulation of p27kip1 inhibits cell proliferation at the G1 to S transition stage of cell cycle, and ultimately, leads to cell apoptosis.

Table 1Annexin V analysis of MyLa cells after 48 h of 25 μ M rexinoid treatment

	Control (%) [#]	UAB30 ^a (%)	Bexarotene (%)
Early apoptotic cells (V ⁺ , PI ⁻)	1.6 $\pm$ 0.5	7.5 $\pm$ 0.7 ^{**}	9.4 $\pm$ 1.1 [*]
Live cells (V ⁻ , PI ⁻)	92 $\pm$ 0.4	76 $\pm$ 1.1 ^{**}	64 $\pm$ 3.8 [*]
Apoptotic cells (V ⁺ , PI ⁺)	3.0 $\pm$ 0.5	6.4 $\pm$ 0.4 [*]	9.6 $\pm$ 1.4 [*]
Necrotic cells (V ⁻ , PI ⁺)	2.7 $\pm$ 0.6	9.5 $\pm$ 1.0 ^{**}	17 $\pm$ 1.4 ^{**}

^aUAB30, 9-cis UAB30[#]average of percent cell population $\pm$ standard deviation

V, annexin V; PI, propidium iodide

^{*}p < 0.001;^{**}p < 0.0001, significantly different from control (DMSO)-treated cells, n = 3.

Table 2Annexin V analysis of HuT 78 cells after 24 h of 25 μ M rexinoid treatment

	Control (% [#])	UAB30 ^a (%)	Bexarotene (%)
Early apoptotic cells (V ⁺ , PI ⁻)	2.2 $\pm$ 1.0	5.4 $\pm$ 1.3 ^{**}	3.3 $\pm$ 1.2
Live cells (V ⁻ , PI ⁻)	94 $\pm$ 1.8	87 $\pm$ 3.8 ^{**}	86 $\pm$ 4.5 ^{**}
Apoptotic cells (V ⁺ , PI ⁺)	3.5 $\pm$ 0.9	6.1 $\pm$ 2.2 [*]	4.2 $\pm$ 0.8
Necrotic cells (V ⁻ , PI ⁺)	0.7 $\pm$ 0.2	1.6 $\pm$ 1.0 [*]	6.5 $\pm$ 4.8 [*]

^aUAB30, 9-cis UAB30[#]average of percent cell population $\pm$ standard deviation

V, annexin V; PI, propidium iodide

^{*}p < 0.001;^{**}p < 0.0001, significantly different from control (DMSO)-treated cells, n = 6.

Table 3Annexin V analysis of Jurkat cells after 24 h of 25 μ M rexinoid treatment

	Control (%) [#]	UAB30 ^a (%)	Bexarotene (%)
Early apoptotic cells (V ⁺ , PI ⁻)	1.1 $\pm$ 0.2	1.6 $\pm$ 0.5	1.3 $\pm$ 0.3
Live cells (V ⁻ , PI ⁻)	95 $\pm$ 0.4	93 $\pm$ 0.8	94 $\pm$ 0.0
Apoptotic cells (V ⁺ , PI ⁺)	3.5 $\pm$ 0.2	4.6 $\pm$ 0.4 [*]	3.7 $\pm$ 0.2
Necrotic cells (V ⁻ , PI ⁺)	0.6 $\pm$ 0.2	0.3 $\pm$ 0.2	0.7 $\pm$ 0.1

^aUAB30, 9-cis UAB30[#]average of percent cell population $\pm$ standard deviation

V, annexin V; PI, propidium iodide

^{*}p < 0.05, significantly different from control cells; n = 3/treatment

Table 4

Ratios of cell cycle stages of MyLa cells treated with rexinoids

Treatment	Time	Average of G1/S	Average Fold ↑
DMSO	48 h	1.10 ± 0.07	1
UAB30 (25 µM)	48 h	2.14 ± 0.29 ^{*%}	1.94 ± 0.26 [%]
Targretin (25 µM)	48 h	1.48 ± 0.14 [*]	1.35 ± 0.12
Treatment	Time	Average of G2/G1	Average Fold ↑
DMSO	48 h	0.13 ± 0.04	1
UAB30 (25 µM)	48 h	0.21 ± 0.05	1.60 ± 0.36
Targretin (25 µM)	48 h	0.13 ± 0.07	0.99 ± 0.56

N = 3/treatment

Ratios are average ± SD (standard deviation)

^{*}p < 0.05, Significantly different from DMSO-treated cells[%]p < 0.05, Significantly different from Targretin-treated cells