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# Synthesis of New Targretin® Analogues that Induce Apoptosis in Leukemia HL-60 Cells

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**Abstract**—Four new analogues of Targretin where the carboxylic acid function was replaced by an *N*,*N*-dimethyl-*S*-aryl carbamate or *N*,*N*-dimethyl-*O*-arylthiocarbamate function, were synthesized. Compounds **5**, **6** and **7** have shown to be more potent than the parent compound to induce apoptosis of HL-60 cells.


## Introduction

Retinoids regulate many important biological processes such as mediation of cell growth and differentiation in both normal and neoplastic cells, and modulation of programmed cell death also known as apoptosis.[1] The broad spectrum of physiological activities of retinoids is mediated by two types of receptors, the retinoic acid receptors (RAR α,β,γ) and the retinoid X receptors (RXR a,b,g). All-*trans* retinoic acid (RA) binds only RAR's, while 9-*cis* RA is an agonist for both RAR's and RXR's.[1,2] These two compounds have a significant implication for the treatment of dermatological diseases, and cancers. They induce remissions in high proportion of patients with acute promyelocytic leukemia (APL).[3] But side effects due to their ability to activate multiple retinoid receptors provide for identifying retinoids receptor selective having specific cancer chemoprotective and chemotherapeutic activities without concomitant unfavorable side effects. Recently, LGD-1069 (Targretin®) (Fig. 1) was identified as a highly selective RXR agonist with low affinity for RAR's. This compound, now in clinical trials, has proven its efficiency for the treatment of mammary adenocarcinome, and cutaneous T-cell lymphoma.[4]

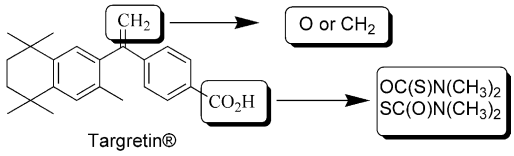


**Figure 1.** Targretin and its synthesized analogues.

The goal of this study was to investigate the influence of new polar heads on the activity of Targretin® against human promyelocytic leukemia HL-60 cell line.[5] It appears interesting to synthesize analogues in which carboxylic acid function was replaced respectively by *O*-arylthiocarbamate and *S*-arylcarbamate moiety (Fig. 1). The different compounds have been evaluated on their capacity to induce apoptosis of leukemia HL-60 cells.

## Chemistry

Synthetic routes to the different analogues are outlined in Figure 2. Following literature protocols,[6] starting derivative **1** was synthesized in high yield. Synthesis of

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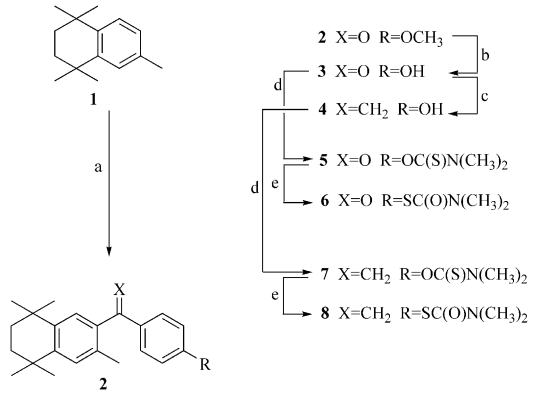


**Figure 2.** Synthesis of the different analogues starting from compound **1**. Reagents and conditions: (a) *p*-methoxybenzoyl chloride, $AlCl_3$ 70%; (b) $BBr_3$, $CH_2Cl_2$, −60 °C, 98%; (c) Tebbe reagent 80%; (d) *N*,*N*-dimethylthiocarbamoyl chloride, DABCO, DMF 70–75%; (e) 200 °C, 8 h, 70–95%.

Targretin analogues was achieved from **1** by straightforward high yielding methods in four or five steps.

The Friedel and Craft acylation of **1** with *p*-methoxybenzoyl chloride provided compound **2**, which after treatment with boron tribromide at −60 °C gave phenol **3** in good yield. Olefination of **3** with Tebbe reagent[7] afforded the derivative **4**. The *S*-arylcarbamate analogues **6** and **8** were synthesized starting from the compounds **3** and **4** using the Newman–Kwart rearrangement.[8] Treatment of **3** and **4** with *N*,*N*-dimethylthiocarbamoyl chloride and DABCO led to the *O*-arylthiocarbamates **5** and **7**. Thermic rearrangement at 200 °C afforded the *S*-aryl carbamate **6** and **8**. All new compounds were fully characterized by usual spectroscopy methods.[9] The *O*-arylthiocabamate and *S*-arylcarbamate can be easily distinguished by $^1H$ NMR spectroscopy. Indeed, the 6 protons of the *N*,*N*-dimethyl moiety appeared as a singlet in the case of *S*-arylcarbamate and as a doublet for the *O*-arylthiocarbamate derivatives when spectra are recorded at room temperature.

## Biological Assays

Compounds **5**, **6**, **7** and **8** were examined for their ability to induce apoptosis on HL-60 cell line.

## Materials and Methods

### Cell line and assessment of apoptosis by flow cytometry

HL-60 cell line (American Type Culture Collection, CCL240 Rockville, USA) is a human myelomonocytic cell line arrested at a myeloblastic stage. The cell line was routinely cultured at 37 °C, 5% $CO_2$ in complete medium (RPMI 1640–10% FCS (Life Technologies, Cergy-Pontoise, France). Cell cultures were checked for the absence of mycoplasma contaminations (Detection kit, Boehringer Mannheim, Meylan, France), and performed without antibiotics to avoid masking silent bacterial and viability (higher than 95%) was determined by trypan blue exclusion. Treatment of cells with 0.02% DMSO as control (a concentration achieved in cells treated with 2 μM retinoids) did not result in any significant effect on inhibition of cell growth or on induction of apoptosis.

Retinoid stock solutions in dimethylsulfoxide were stored at −20 °C. Cells treated with retinoids (2 μM) were seeded at a density of $2\times10^5$ cells/mL and were collected 4 days or 7 days later.

For hipodiploid measurement, cells ($2.10^6$ cells/assay) were washed twice in PBS and permeabilized with 75% ethanol for 2 min at 4 °C. The cells were incubated overnight at 4 °C with propidium iodide (40 μg/mL) and RNAse A (100 μg/mL) in PBS. Flow cytometric analysis of retinoid-treated cells was done on a FACSCAN flow cytometer. Fluorescence data are expressed by percent of cells. Apoptotic cells were the cells with a fluorescence value down to the Go/G1 pic. Research products: culture media were from Life Technologies.

### Detection of Apoptosis by Confocal Microscopy

Four days after addition of retinoid analogues, cells were collected using Cytospin cell preparation system (Shandon, Pittsburgh, PA) and TUNEL stained according to the manufacturer recommendations (Boehringer Mannheim). Positive controls were performed by incubation of HL-60 cells with 0.5 mg/mL of DNAse1 (Sigma) at room temperature for 10 min prior TUNEL staining. DNAse1-treated cells incubated with fluorescein-labeled nucleotide mixture, without addition of terminal deoxynucleotidyl transferase (TdT), were used as negative controls. The TUNEL-labelled cells were also stained with DAPI (in order to visualize all nuclei) and TRITC-phalloidin (for F-actin detection). The slides were observed in a Leica TCS 4D Laser Confocal Microscope. Fluorescence confocal images (TUNEL and F-actin) were acquired simultaneously using a 25X NA 0.75 Phaco 2 oil-immersion lens. In some cases DAPI fluorescence images for the same microscope field were also recorded using a Hitashi KP-161 CCD camera.

### Quantitative Analysis

Three fields for each experimental condition were chosen by random sampling and examined by fluorescence microscopy (F-actin, red fluorescence and TUNEL labelling, green fluorescence). The total number of cells (F-actin labelling) and the number of TUNEL positive nuclei were counted on digitized images using Image Tool software. The results were expressed as percent of the estimated total number of cells present in the field. The main advantage of this method is in the significance of the results that can be achieved. Our previous work has proven that this methodology is reliable even with very low level of apoptosis ($<1\%$).[10]

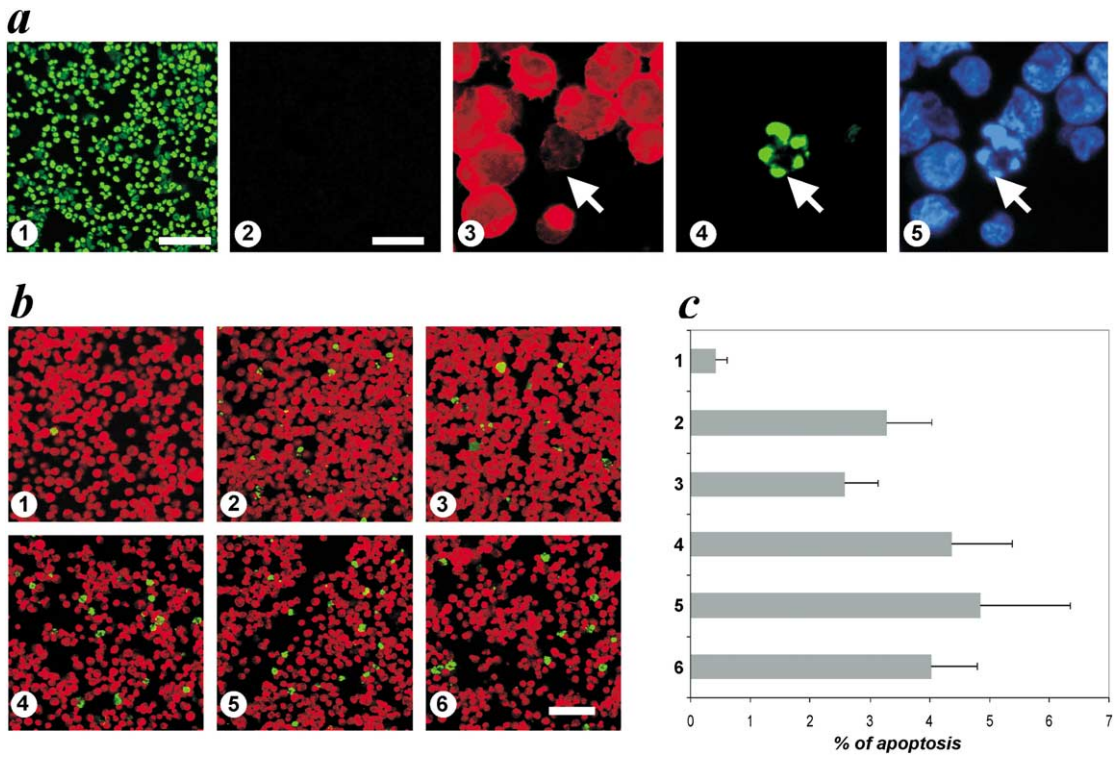


**Figure 3.** (a) Changes in the morphology of nucleus and actin fibers during apoptosis induced by TA (compound **6**). 3, Phalloidin staining; 4, TUNEL staining; 5, DAPI staining. 1 and 2 represent TUNEL positive and negative control, respectively. Scale bars: 100 μm (1, 2); 10 μm (3–5); (b) induction of genomic DNA-nicking in apoptotic HL-60 cells treated with 2 μM Targretin (T) or Targretin analogues (TA) for 4 days. Cytospins were prepared from control (1), T (2) or TA-treated cells (3: compound **8**, 4: compound **7**, 5: compound **6**, 6: compound **5**), and the TUNEL assay counterstained with rhodamine-phalloidin. Labeling of the apoptosis-specific DNA strand breaks appears in green, F-actin in red (scale bar, 70 μm); (c) estimation of apoptosis using quantitative confocal microscopy (1: control, 2: Targretin, 3: compound **8**, 4: compound **7**, 5: compound **6**, 6: compound **5**).

## Results and Discussion

The therapeutic actions of retinoids are due to their ability to regulate cellular process such as cellular differentiation, proliferation and modulation of apoptosis.[1] To test if new synthesized Targretin analogues induce apoptosis in HL-60 leukemia cells, DNA fragmentation analysis was performed. HL-60 cells incubated in defined media and treated with 2 μM of Targretin (T) or Targretin analogues (TA) demonstrated a clear induction of apoptosis when analyzed by TUNEL labeling and propidium iodide incorporation. These results have been obtained through two independent methods: quantitative confocal microscopy (Fig. 3b and c) and flow cytometry (Fig. 4), were conducted under a double blind protocol. Fluorescence microscopy observation of T and TA-treated HL-60 cells showed typical characteristics of apoptosis including morphological change of actin microfilament[11] (Fig. 3, a3 arrow), genomic DNA cleavage (Fig. 3, a4 arrow) and nuclear fragmentation (Fig. 3, a5 arrow). Control cells growing exponentially showed very limited TUNEL labeling (Fig. 3, b1), whereas T and TA-treated cells showed numerous TUNEL positive cells (Fig. 3b 2–6). The most potent inducer's of apoptosis is the compound **6**. In addition, the compounds **7**, **6** and **5** induce levels of apoptosis significantly higher ($P < 0.05$) than the targretin while the compound **8** is less effective (Fig. 3c). These quantitative results were confirmed by flow cytometry (Fig. 4) and were consistent with the levels of apoptosis previously found in HL-60 cells treated with other retinoid analogues.[6]

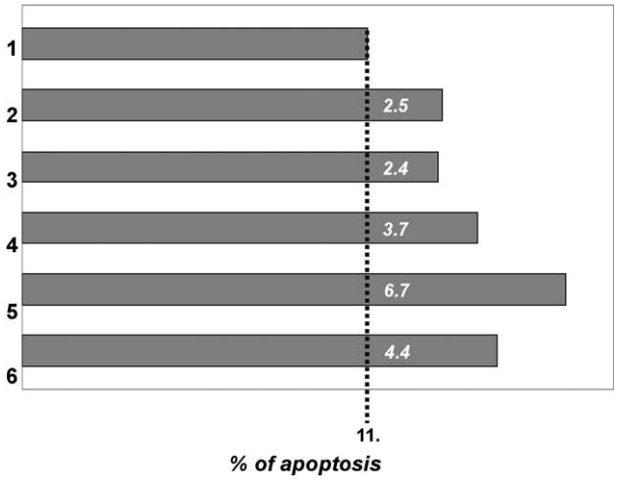


**Figure 4.** Estimation of apoptosis using flow cytometry (1) control, (2) Targretin, (3) compound **8**, (4) compound **7**, (5) compound **6**, (6) compound **5**.

## Conclusion

In summary, we report the synthesis of new analogues of Targretin. The replacement of the carboxylic function by *N*,*N*-dimethylcarbamate or *N*,*N*-dimethylthiocarbamate functions led to compounds which induce apoptosis of HL-60 cells in higher level than the parent compound. We show that the polar carboxylic function which was thought to be essential for activity[1,2] could be replaced by non polar functions leading to more potent compounds. These preliminary results are quite encouraging and make compounds **5**, **6**, and **7** good candidates for further biological investigations. This work is currently underway to determine by which nuclear receptors are mediated the effects of these compounds and will be reported in due courses.

## Acknowledgements

Ministère de l'Enseignement Supérieur et de la Recherche (doctoral fellowship J-Y.W.), Association de Recherche sur le Cancer (ARC Grants Nos. 9519 and 5761), and the Laboratoires Mayoly Spindler are gratefully acknowledged for support of this work.

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9. All new compounds were characterized by $^1$H NMR and FAB mass spectrometry. Elemental values (C, H, O) were within 0.4% of the theorical values. $^1$H NMR data of selected compounds: Compound **5**: $^1$H NMR (300 MHz, $CDCl_3$) δ 7.9 (d, $J=12$ Hz, 2H), 7.25 (s, 1H), 7.20 (s, 1H), 7.15 (d, $J=12$ Hz, 2H), 3.4–3.5 (2s, 6H), 2.35 (s, 3H), 1.7 (s, 4H), 1.3 (s, 6H), 1.2 (s, 6H). Compound **7**: $^1$H NMR (300 MHz, $CDCl_3$) δ 7.35 (d, $J=8$ Hz, 2H), 7.15 (s, 1H), 7.05 (s, 1H), 6.95 (d, $J=8$ Hz, 2H), 5.75 (d, $J=2$ Hz, 1H), 5.2 (d, $J=2$ Hz, 1H), 3.35–3.5 (2s, 6H), 2 (s, 3H), 1.7 (s, 4H), 1.3 (s, 6H), 1.25 (s, 6H). Compound **6**: $^1$H NMR (300 MHz, $CDCl_3$) δ 7.85 (d, $J=8$ Hz, 2H), 7.6 (d, $J=8$ Hz, 2H), 7.25 (s, 1H), 7.2 (s, 1H), 3.1 (s, 6H), 2.3 (s, 3H), 1.75 (s, 4H), 1.3 (s, 6H), 1.2 (s, 6H). Compound **8**: $^1$H NMR (300 MHz, $CDCl_3$) d 7.45 (d, $J=8$ Hz, 2H), 7.3 (d, $J=8$ Hz, 2H), 7.15 (s, 1H), 7.1 (s, 1H), 5.8 (d, $J=2$ Hz, 1H), 5.25 (d, $J=2$ Hz, 1H), 3.1 (s, 6H), 2 (s, 3H), 1.7 (s, 6H), 1.25 (s, 6H).
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