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Synthesis and proapoptotic activity of oleanolic acid derived amides

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ABSTRACT

Thirty-one different 3-*O*-acetyl-**OA** derived amides have been prepared and screened for their cytotoxic activity. In the SRB assays nearly all the carboxamides displayed good cytotoxicity in the low μM range for several human tumor cell lines. Low EC_{50} values were obtained especially for the picolinylamides **14–16**, for a *N*-[2-(dimethylamino)-ethyl] derivative **27** and a *N*-[2-(pyrrolinyl)-ethyl] carboxamide **28**. These compounds were submitted to an extensive biological testing and proved compound **15** to act mainly by an arrest of the tumor cells in the S phase of the cell cycle. Cell death occurred by autophagy while compounds **27** and **28** triggered apoptosis.

1. Introduction

The search for effective treatments of cancer is a continuing quest since the first written record of this disease reported as early as circa 1600 BCE (Edwin Smith papyrus). One of the several treatments is chemotherapy, and many chemotherapeutics have been developed so far. Many of them were gained from secondary natural products, for example Vinca-alkaloids (from poisonous evergreen, *Catharantus roseus*) or taxoles (diterpenes first derived from the Pacific yew tree, *Taxus brevifolia*). In addition, it has been postulated that a regular consumption of fruits and herbs helps reducing the carcinogenic risk [1,2].

Oleanolic acid (**OA**) is a pentacyclic triterpenoic acid that is widespread in different plants. This valuable compound has been detected and isolated from many fruits, for example from cranberries [3], apples [4] and olives [5–7]. Many biological activities have been credited [8] to **OA**, such as an anti-inflammatory, antitumor, antioxidant effect, hypoglycemic as well as hepatoprotective properties [9,10], and **OA** seems also to prevent progressing of cardiovascular diseases [11]. Of special interest are its cytotoxicity and its antiproliferative properties of **OA**. During the last year alone many publications dealt with different effects of **OA** on tumor cells and its ability to suppress the proliferation of human bladder cancer [12] and of human prostate cancer cells *in vivo* and *in vitro* [13]. **OA** also triggers an arrest of the cell cycle and apoptosis in human hepatocellular carcinoma cells [14].

In the context exploring the chemo-preventive effects of **OA** (as an ingredient of grapes and olives) [15] in human breast cancer, Sanchez-Quesada et al. [6,7] were able to show the inhibitory impact of **OA** on the proliferation of human breast adenocarcinoma cells (MCF-7). All these examples not only prove the potential of secondary natural products, but also reflect the impact of **OA** against cancer cells. These findings make **OA** a promising lead compound for developing new cytotoxic/antitumor active compounds.

Results from several groups have demonstrated that structural modifications of **OA** have a high impact onto the biological activity [16–20]. Keeping in mind previous findings for enhancing the cytotoxic properties of triterpenoic acids [21–24] but also economic aspects, we decided to investigate modifications at position C-28 of **OA** in more detail. Based on an analysis of the Comprehensive Medicinal Chemistry Database >25% of known drugs contain a carboxamide as a structural feature [25]. This fact supported the decision the synthesis of triterpenoic carboxamides might be most rewarding to find new cytotoxic agents for treating cancer.

An **OA** derived amide was first described as early as 1937 by Ruzicka et al. [26] gained from the reaction of **OA** with thionyl chloride followed by adding of ammonia. This synthetic approach was extended. In the years to follow several **OA** derived amides

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have been synthesized [20,27–29]. Their biological importance, however, remained unexplored for many years, and to our knowledge, a first investigation of the cytotoxic potential of **OA** derived amides (employing ω-amino esters) has been performed by Assefa et al. in 1999 [30]. It was the merit of Gupta et al. [31] to show the improved cytotoxicity of **OA** anilides, while introducing long-chain alkyl carboxamides lowered the cytotoxicity of triterpenoic acids. Up to now, however, little is known about the structure-activity relationships of **OA** carboxamides. Hence we set out to synthesize 31 different analogs starting from 3-*O*-acetyl-oleanolic acid (**1**) – a well accessible starting material. More investigations about the mechanism of cell death induced by these carboxamides were performed.

2. Experimental part

2.1. General

Melting points are uncorrected (*Leica* hot stage microscope), NMR spectra were recorded using the Varian spectrometers Gemini 2000 or Unity 500 (δ given in ppm, *J* in Hz, internal Me_4Si; typical experiments: H-H-COSY, HMBC, HSQC, NOESY, DQF-COSY), MS spectra were taken on a Finnigan MAT LCQ 7000 (electrospray, voltage 4.1 kV, sheath gas nitrogen) instrument. The optical rotations were measured on a Perkin-Elmer polarimeter at 20 °C; TLC was performed on silica gel (Merck 5554, detection with cerium-molybdate reagent); elemental analyses were performed on a Vario EL (CHNS). IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Spectrum 1000. Fluorescence microscopic images were recorded on an Axioskop 20 with an AxioCam MR3 (Carl Zeiss AG). Flow cytometric experiments were performed on an Attune acoustic focusing cytometer (Life Technologies GmbH). The solvents were dried according to usual procedures. The purity of the compounds was determined by HPLC and found to be >98%. Oleanolic acid (**OA**) was obtained from different commercial suppliers in bulk quantities.

2.2. Biology

2.2.1. Cell lines and culture conditions

The cell lines used are human cancer cell lines: 518A2 (melanoma), A2780 (ovarian carcinoma), HT29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), A549 (alveolar basal epithelial adenocarcinoma), 8505C (thyroid), and non-malignant mouse fibroblasts NIH 3T3. Cultures were maintained as monolayers in RPMI 1640 medium with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2.2. Cytotoxicity assay (SRB assay)

The cytotoxicity of the compounds was evaluated using the sulforhodamine-B (Kiton-Red S, ABCR) micro culture colorimetric assay. Cells were seeded into 96-well plates on day 0 at appropriate cell densities to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with six different concentrations (1, 3, 7, 12, 20 and 30 μM) minimum. The final concentration of DMSO/DMF never exceeded 0.5%, which was non-toxic to the cells. After a 96 h treatment, the supernatant medium from the 96-well plates was discarded, the cells were fixed with 10% trichloroacetic acid (TCA) and allowed to rest at 4 °C. After 24 h fixation, the cells were washed in a strip washer and dyed with SRB solution (100 μl, 0.4% in 1% acetic acid) for about 20 min. After dying, the plates were washed four times with 1% acetic acid to remove the excess of the dye and allowed to air-dry overnight. Tris base solution (200 μl, 10 mM) was added to each well and absorbance was measured at $\lambda = 570$ nm using a 96 well plate reader (Tecan Spectra, Crailsheim, Germany). The EC_{50} values were averaged from three independent experiments performed each in triplicate calculated from semi logarithmic dose response curves applying a non-linear 4P Hills-slope equation (GraphPad Prism5; variables top and bottom were set to 100 and 0, respectively).

2.2.3. AO/PI dye exclusion assay

Morphological characteristics of cell death were analyzed employing an AO/PI dye exclusion assay using human cancer cell line A2780. Approx. $8 \cdot 10^5$ cells were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow up for 24 h. After removing of the used medium, the substance loaded fresh medium was reloaded (or a blank new medium as a control). After 24 h, the content of the flask was collected and centrifuged (1200 rpm, 4 °C), the pellet was gently suspended in phosphate-buffered saline (PBS (w/w), 1 mL) and centrifuged again. The PBS was removed, and the pellet gently suspended in PBS (50 μl) again. The analysis of the cells was performed using a fluorescence microscope after having mixed the cell suspension (10 μl) with a solution of AO/PI (1 μg/ml, 10 μl).

2.2.4. Detection of acidic compartments-AO- and MDC-staining

Formation of acidic vesicles and autolysosomes was analyzed using acridine orange (AO) respectively monodansylcadaverine (MDC). The cells were prepared in the same manner as described above. The analysis of the cells was performed using a fluorescence microscope after having mixed the cell suspension (10 μl) with a solution of AO (1 μg/ml, 10 μl) respectively MDC (50 μg/ml, 10 μl).

2.2.5. Flow cytometry

The following assays were performed by same sample preparation. Approximately $8 \cdot 10^5$ cells (A2780) were seeded in cell culture flasks (25 cm^2), and the cells were allowed to grow up for 24 h. After removing of the used medium, the substance loaded fresh medium was reloaded (or a blank fresh medium as a control). After 24 h further sample preparation was conducted by protocols reported below.

2.2.5.1. Cell cycle investigations. For cell cycle investigations the adherent cells were harvested (except for **27** and **28**, there the whole content of the flask was also collected), centrifuged (1200 rpm, 4 °C), and washed twice with PBS ((w/w), 1 mL). The cells were counted and approximately $1 \cdot 10^6$ cells were fixed with ethanol (70%, 4 °C, 24 h). After centrifugation (1200 rpm, 4 °C) the cells were washed with staining buffer (PBS (w/w), containing 2% FCS and 0.01% NaN_3 (2% in H_2O), 1 mL) and centrifuged. The pellet was gently suspended in RNAase A (100 μl, 100 mg/ml) and incubated for 30 min at 37 °C. After re-suspending cells in 1 mL PI buffer (20 μl PI solution (1 mg/mL) in staining buffer) and incubating for 30 min at room temperature in the dark, cells were analyzed using the Attune® FACS machine; collecting data from the BL-2A channel. Doublet cells were excluded from the measurements by plotting BL-2A against BL-2H. For each cell cycle distribution 20,000 events were collected in technical triplicates, each sample was measured in duplicates. Cell cycle distribution was calculated using ModFitLT™ (Verity Software House, Topsham, US).

2.2.5.2. Annexin V/PI assay. The content of the flask was collected and centrifuged (1200 rpm, 4 °C). The pellet was gently suspended in phosphate-buffered saline (PBS (w/w), 1 mL), centrifuged, and washed twice with PBS ((w/w), 1 mL). The cells were counted