Antiproliferative Activity and Effect on GABA_A Receptors of Callitrisic Acid Derivatives

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ABSTRACT
The semisynthesis and biological activity of the naturally occurring abietane diterpenoids callitrisic acid (4a; 4-epidehydroabietic acid) and callitrisinol (6) are reported. These compounds and jiadifenoic acid C (5) were obtained from methyl callitrisate (4b) isolated from Sandarac resin for biological evaluation and comparison with the biological activities of C4 epimers such as dehydroabietic acid (2a). In particular, the antiproliferative activity against a panel of six representative human solid tumor cell lines (A549, HBL-100, HeLa, SW1573, T-47D, WiDr) and the effect on GABA_A receptors (α1β2γ2s) were evaluated. The GI₅₀ values were in the range of 3.4–61 µM and the potentiation of I_GABA was 269–311 % at 100 µM. Callitrisinol (6) was found to be 6.7-fold more potent than the reference etoposide in the WiDr (colon) cancer cell line. The role of the stereogenic center at C4 for antiproliferative and GABA_A receptor modulating activities in the dehydroabietane scaffold has thus been revealed.

Introduction
At present, about fifty percent of commercial pharmaceutical drugs are derived from natural sources [1]. The abietane-type and related diterpenoids are a class of naturally occurring terpenoids in the plant kingdom, which have demonstrated a wide range of biological activities against cancer and a variety of infectious diseases (viral and bacterial) [2]. Several research groups have explored the potential as chemotherapeutic agents of abietanes by means of semisynthetic derivatives from abietic acid (1a)-derived materials such as dehydroabietic acid (2a, DHA) and dehydroabietylamine (3), also called leelamine (Fig. 1) [3]. For example, DHA displays not only antiulcer and antimicrobial properties, but also antitumor effects [2, 3]. Recently, DHA was reported as a positive GABA_A receptor modulator inducing significant receptor modulation in the oocyte assay, with a maximal potentiation of I_GABA of 682.3 ± 44.7 % at 100 µM [4].

DHA displays an equatorial carboxylic group located at C18, while in other natural congeners, the carboxylic group adopts the axial configuration (C19) as in 4-epidehydroabietic acid or callitrisic acid (4a; Fig. 1). Callitrisic acid (4a) is a diterpenoid acid contained in the resins of several Callitris species, a small genus of the family Cupressaceae, mostly found in Australia though also present
Methyl callitrisate (4b). Thus, nucleophilic methyl ester cleavage of 4b with LiI afforded callitrisic acid (4a) in an 84 % yield, while reduction with LiAlH₄ gave callitrisinol (6) in a 96 % yield (> Fig. 2). Jiadifenoic acid C (5) was prepared following our method [9] by regioselective dehydrogenation of methyl callitrisate (4b) with 2,3-dichloro-5,6-dicyanoquinone (DDQ), followed by allylic oxidation with catalytic selenium dioxide and tert-butyl hydroperoxide (TBHP) as a co-oxidant and, finally, methyl ester cleavage with LiI to afford jiadifenoic acid C (5) in a 22 % overall yield (> Fig. 2).

With compounds 4-6 in hand, the antiproliferative activity against six representative human solid tumor cell lines, A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), T-47D (breast), and WiDr (colon), was studied using the sulforhodamine B (SRB) assay [14]. The results expressed as GI₅₀ are given in > Table 1. The standard anticancer drugs etoposide and cisplatin were used for comparison. All compounds were active (GI₅₀ < 100 µM) in the cell lines tested, with jiadifenoic acid C (5) being the least potent compound with GI₅₀ values in the range of 19–61 µM against all cell lines, while compounds 4b and 6 were the most potent at a similar level. In particular, compound 6 was the most potent against WiDr cells (GI₅₀ = 3.4 µM), while compound 4b was the most potent against T-47D cells (GI₅₀ = 8.8 µM), being 6.7- and 2.5-fold more potent than the reference compound etoposide, respectively. In general, the order of activity in the callitrisic series with different functional groups at C19 was alcohol ≥ ester > acid. Our previous study on the biological activity of dehydroabietic acid (2a) derivatives (HeLa and Jurkat cell lines) was also consistent with this order of activity [15]. Though the GI₅₀ values obtained with the SRB assay and IC₅₀ values obtained with the MTT assay are not fully comparable, it is worth to note that the dehydroabietic acid derivatives (C18-functionalized) in our previous study [15] gave IC₅₀ values in the range of 45–337 µM for HeLa cells. This fact involved less active compounds in the dehydroabietic series. In order to confirm this activity tendency, the GI₅₀ values for dehydroabietic acid (2a) and its methyl ester (2b) in A549, HBL-100, HeLa, SW1573, T-47D, and WiDr cells were obtained and compared with the values of the semisynthetic callitrisic acid (4a) series. Thus, in general, it can be concluded that our C19-functionalized callitrisic series (4a, 4b) was more potent than the corresponding C18-functionalized series. Also, a SAR trend is that a C19 hydroxymethyl group produced the best antiproliferative activity, whereas a C19 carboxylic group led to less active compounds, including jiadifenoic acid C (5) as the least potent. Thus, the presence of an allylic alcohol at C13 seems to be detrimental for the antiproliferative activity.

In another experiment based on the oocyte assay, compounds 4-6 were tested for their effects on GABA_A receptors (α₂β₂γ₂) by means of the two-electrode voltage clamp technique in Xenopus laevis oocytes [4]. All compounds were screened at concentrations of 10 and 100 µM and compared with DHA (2a) as a positive control [4]. The results are summarized in > Table 2. Callitrisinol (6) modulated IC₅₀ of 116 % and 311 %, respectively, while methyl callitrisate (4b) and jiadifenoic acid C (5) were inactive. Callitrisic acid (4a) only enhanced GABA evoked currents at 100 µM (potentiation of IC₅₀ of 269 %). Thus, it can be concluded that the presence of an allylic alcohol at C13 significantly reduced the GABA_A receptor modulating effect, while the presence of a C19 hydroxymethyl group increases activity. On comparing GABA_A modulating activity of DHA (2a; potentiation of IC₅₀ of 789 % at 100 µM) with its C4-epimer, callitrisic...
acid (4a; potentiation of $I_{\text{GABA}}$ of 269% at 100 µM), it can be concluded that the stereochemistry at C4 is very important for $GABA_A$ activity, with the callitrisic acid series being the least active.

In summary, the compounds in this communication support the importance of the aromatic abietanes with a dehydroabietane skeleton for antiproliferative activity. Therefore, these abietane compounds may be useful leads for the development of novel antitumor drugs. The role of the stereogenic center at C4 for antiproliferative and $GABA_A$ receptor modulating activities in the dehydroabietane scaffold have been revealed. Further studies to identify more structure-activity relationships and enhance the observed activities are under way.

### Materials and Methods

#### General experimental procedures

Optical rotations were measured using a 5-cm cell in a Schmidt-Haensch Polartronic-D polarimeter. NMR spectra were recorded on a 300 MHz spectrometer. All spectra were recorded in CDCl$_3$ as the solvent unless otherwise stated. Complete assignments of $^{13}$C NMR multiplicities were made on the basis of DEPT experiments. J values are given in Hz. MS data were acquired on a QTOF spectrometer. Reactions were monitored by TLC using Merck silica gel 60 F-254 in 0.25-mm thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10% sulfuric acid solution and heating with a heat gun. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). Commercial reagent grade solvents and chemicals were used as purchased unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated under reduced pressure.
Compounds
Compounds 1, 2, 4b, and 5 were prepared according to known procedures [9, 15]. Compound 4b was used as starting material for the preparation of compounds 4a and 6. All compounds prepared in this work exhibit spectroscopic data in agreement with the proposed structures. The purity of all final compounds was 95 % or higher.

Antiproliferative assay
Cells were inoculated onto 96-well microtiter plates in a volume of 100 µL per well at densities of 2500 (A549, HBL-100, and HeLa) and 5000 (SW1573, T-47D, and WiDr) cells per well, based on their doubling times. Compounds 1-6 were initially dissolved in DMSO at 400 times the final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25 % v/v, negative control). Each compound was tested in triplicate at different concentrations which cells were precipitated with 25 µL of ice-cold TCA (50 % w/v) and fixed for 60 min at 4 °C. Then, the SRB assay was performed as previously described [14]. The optical density (OD) of each well was measured at 530 nm using BioTek’s PowerWave XS absorbance microplate reader. Values were corrected for background OD from wells containing only medium. The antiproliferative activity for each compound, expressed as GI50 values, was calculated according to NCIC formularas [14].

Expression of GABAA receptors in Xenopus laevis oocytes
The experiments were carried out following procedures formerly described with a few modifications [4, 16]. Briefly, follicle membranes covering oocytes were enzymatically digested with 2 mg · mL⁻¹ collagenase (type 1A). The coding regions of plasmids were sequenced before experimental use. After cDNA linearization, capped cRNA transcripts were produced using the mMESSAGE mACHINE® T7 transcription kit (Life Technologies). Capped transcripts were polyadenylated using yeast poly(A)polymerase, diluted in nuclease-free water, and stored before injection at -80 °C.

One day after isolation, the oocytes were injected with about 10–50 nL of nuclease-free water containing the different rat cRNAs (100–2000 ng · µL⁻¹ per subunit). For expression of wild-type α1β2γ2S, cRNAs were mixed in a ratio of 1:1:10. Electrophysiological experiments were performed using the two-microelectrode voltage clamp technique at a holding potential of −70 mV, making use of a TURBO TEC 01C amplifier (NPI Electronic) and an Axon Digidata 1440A interface (Molecular Devices). Data acquisition was carried out using pCLAMP v.9.2 (Molecular Devices). The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 5 mM HEPES (adjusted to pH 7.4 using 1 M NaOH). Microelectrodes were filled with 2 M KCl and had resistances between 1 and 3 MΩ.

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Conflicts of Interest
The authors declare no conflict of interest.

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