

Article

Betulin, a newly characterized compound in *Acacia auriculiformis* bark, is a multi-target protein kinase inhibitor

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Abstract: The purpose of this work is to investigate the protein kinase inhibitory activity of constituents from ethyl acetate soluble fraction of *Acacia auriculiformis* stem bark. Column chromatography, gel filtration and NMR spectroscopy were used to purified and characterized betulin from the extract. Betulin which is a known inducer of apoptosis was screened against a panel of 16 disease-related protein kinases. Betulin was shown to inhibit Abelson murine leukemia viral oncogene homolog 1 (ABL1) kinase, casein kinase 1 ϵ (CK1 ϵ), glycogen synthase kinase 3 α/β (GSK-3 α/β), Janus kinase 3 (JAK3), NIMA Related Kinase 6 (NEK6) and vascular endothelial growth factor receptor 2 kinase (VEGFR2) and with activity in μM range. The effect of betulin on the cell viability of doxorubicin-resistant K562R chronic myelogenous leukemia cells was then verified to underline its putative use as anti-cancer compound. Betulin was shown to modulate the mitogen-activated protein (MAP) kinase pathway similarly to imatinib mesylate, a well-known inhibitor of ABL1 kinase. The interaction of betulin and ABL1 was studied by molecular docking showing an interaction of the inhibitor with the ATP binding pocket. Altogether, these data demonstrate that betulin is a multi-target inhibitor of protein kinases, an activity that can contribute to the anticancer properties of the natural compound and notably for treatment of leukemia.

Keywords: triterpenoids / *Acacia* stem bark / polypharmacology / protein kinase inhibitors

1. Introduction

Terrestrial plants are still a crucial source of medicines especially in developing countries. According to the WHO, about 80% of the world's population depends on plant-derived medicines for their health care[1,2]. Secondary metabolites present in natural extracts purified from plants and microorganisms are known to possess multiple bioactivities (ranging from cytotoxic to cytoprotective) and are generally considered to be inexpensive[3]. Accordingly, intensive study has been devoted to the purification and chemical characterization of active constituents which could possibly yield a novel chemical compound suitable for drug development[4,5]. Human protein kinases represent the third largest enzyme class and are responsible for modifying up to one-third of the human proteome. Over 518+ protein kinases that include serine/threonine and tyrosine kinase are known to be encoded by the human genome[6]. Deregulation of kinase function (e.g. by hyperactivation, or mutation) plays an important role in several diseases such as cancer[7], neurodegenerative disorders, inflammation and diabetes, thereby making them attractive targets to the pharmaceutical industry[8]. From 20 to 33% of current drug discovery efforts worldwide are focused on the protein kinase super family[9]. Consequently, the US FDA has already approved 62 small molecule protein kinase inhibitors[10-12]. At least 18 of these drugs are multi-target inhibitors[9]. Metabolites from plants are known to be a rich source of putative protein kinase inhibitors (e. g. flavonoid compounds that function as competitive inhibitors of ATP binding[13]).

The genus *Acacia* belongs to the family Fabaceae and includes about 1400 species of trees and shrubs widespread, throughout warm and semiarid regions of the world including subtropical and tropical Africa (e.g. Nigeria, Senegal, Egypt, and Mozambique)[14]. Within this vast genus, *Acacia auriculiformis*, commonly referred to as Black Wattle, is an important medicinal plant. The Ibibio community of Niger Delta region in Nigeria uses this plant as antimalarial[15]. Moreover, an infusion of the bark of this plant is used to treat inflammation among the aborigines of Australia[16]. Several *Acacia* species including Black Wattle are also known to contain components that inhibit tumor growth, and thus the mechanism for the reported activity is of great interest[17]. In addition, the antimutagenic and chemoprotective activities of *Acacia auriculiformis*, particularly the tannins contained in the bark, as well as the ability of its ethyl acetate and acetone extracts to scavenge free radicals have been reported[18-20]. Recent publications have shown the purification of a new triterpenoid trisaccharide, three new triterpenoids, and have shown antimicrobial activity of acaciaside a and acaciaside b[21,22]. The mosquito larvicidal activity of the fruit extracts and protein kinase inhibitory activity of a tetrahydroxy flavone isolated from the stem bark of *Acacia auriculiformis* have also been reported[23,24].

In the present study, we report the isolation of the triterpenoid betulin and the investigation of this compound's activity against a panel of disease-related kinases. We also demonstrate the effect of betulin on the viability of doxorubicin-resistant and -sensitive human leukemia cell lines.

2. Results

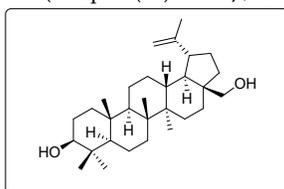
2.1. Purification of betulin from *Acacia auriculiformis* stem bark and biological evaluation against disease-related kinases

A compound was isolated from *Acacia auriculiformis* stem bark as a white amorphous solid. Its spectral properties (^1H and ^{13}C) are consistent with literature data for betulin (3-lup-20(29)-ene-3 β ,28-diol)[25]. *Acacia auriculiformis* stem bark was found to contain about 0.002% of betulin by dry weight.

As triterpenoids have already been shown to have activity against kinases, we tested its inhibitory effect on a panel of protein kinases (PKs). Eight disease-related human PKs were tested including cyclin-dependent kinases (CDK5/p25 and CDK9/CyclinT), Haspin, proto-oncogene proviral integration site for moloney murine leukemia virus-1 (Pim1), glycogen synthase kinase-3 beta (GSK-3 β), casein kinase 1 (CK1 ϵ), Janus kinase 3 (JAK3) and Abelson murine leukemia viral oncogene homolog 1 (ABL1). Table 1 shows the results of the primary screening. For example, betulin showed a weak activity against Pim1; at 10 $\mu\text{g/ml}$ it showed only 10% inhibition of kinase activity. This was contrasted markedly with ABL1 whose kinase activity betulin inhibited by 79%. JAK3 and GSK-3 β were also shown to be inhibited by betulin.

Table 1. Primary screening of Betulin purified from *Acacia auriculiformis* against a panel of eight disease-related human protein kinases.

Betulin (3-lup-20(29)-ene-3 β ,28-diol)



Betulin tested at:	CDK5/ p25	CDK9/ CyclinT	Haspin	Pim1	GSK-3 β	CK1 ϵ	JAK3	ABL1
10 $\mu\text{g/mL}$	99	60	91	90	30	48	25	21
1 $\mu\text{g/mL}$	94	88	72	≥ 100	38	53	28	36

The table reports the results of primary screening performed with 10 $\mu\text{g/mL}$ or 1 $\mu\text{g/mL}$ of betulin. The chemical structure of betulin is depicted above the table. Data are expressed as % of activity remaining, following treatment of each kinase with 10 or 1 $\mu\text{g/mL}$ of the compound, compared to control (DMSO vehicle only) assay. ATP concentration used in the kinase assays was 10 μM (values given represent mean, n=2). ≥ 100 indicates that the compound does not detectably inhibit the enzymatic activity at the tested concentration.

Following this primary screening, betulin was next tested against a larger panel of 16 protein kinases (results obtained are reported in Figure 1). Betulin was tested over a range of concentrations and IC₅₀ values were determined from the dose-response curves for the most potently affected targets (displaying more than ~45% of inhibition at 10 μM of betulin). The results revealed that betulin gave the highest activity against GSK-3 α with IC₅₀ of 0.72 μM followed by ABL1 with IC₅₀ of 0.93 μM (see Figure 2b for the dose-response curve on ABL1), GSK-3 β with IC₅₀ of 1.06 μM , JAK3 kinase with IC₅₀ of 1.08 μM , CK1 ϵ with IC₅₀ of 2.11 μM , vascular endothelial growth factor receptor 2 (VEGFR2) with IC₅₀ of 2.45 μM and NIMA Related Kinase 6 (NEK6) with IC₅₀ of 3.02 μM .

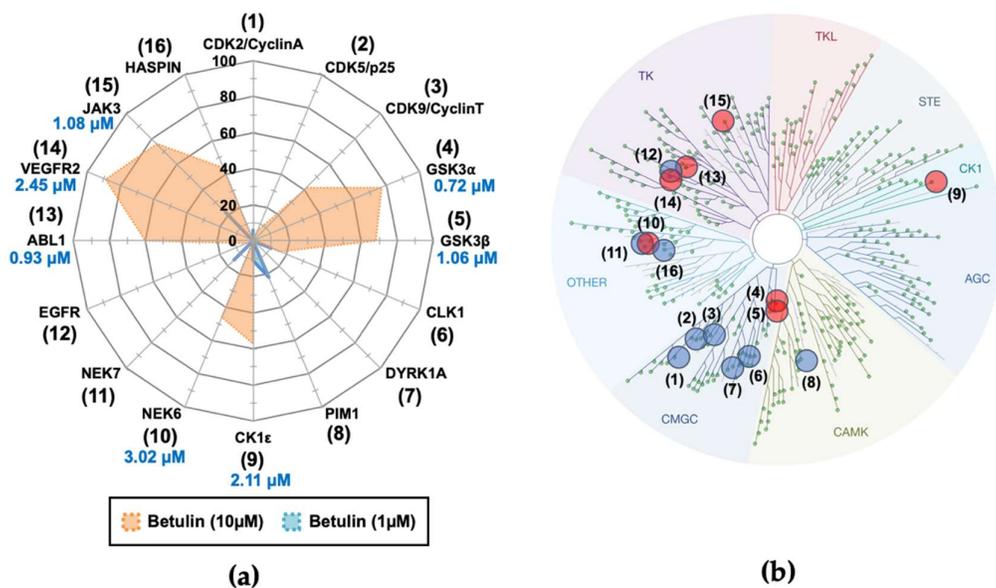


Figure 1. Selectivity of betulin against a panel of 16 disease-related protein kinases. (a) The value reported on the Kiviatic chart are mean (n=2) expressed in % of inhibition, compared with a DMSO control. The IC₅₀ values in μM are listed below the name of each tested kinase. As betulin was not abundant in *Acacia bark*, we used a commercially-available betulin, provided by Sigma (reference #B9757). All protein kinases used here are human except DYRK1A (*Rattus norvegicus*) and CLK1 (*Mus musculus*). (b) The targets used here are selected from the human kinome as represented in the panel by blue dots on the circular tree. Red dots indicate that the kinase is inhibited by betulin. This image was generated using the TREEspot™ Software Tool (Eurofins DiscoverX Corporation, Fremont, CA, USA) and reprinted with permission from KINOMEscan®, a division of Eurofins DiscoverX Corporation (© DiscoverX Corporation 2010). The codes reported on this figure indicate the subclasses of protein kinases: CMGC for CDKs, MAP kinases, GSK, and CDK-like kinases; AGC for protein Kinase A, C, and G families (PKA, PKC, PKG); CAMK for Ca²⁺/calmodulin-dependent protein kinases; CK1, Cell/Casein Kinase 1; STE, STE Kinases (Homologs of yeast STERile kinases); TKL, Tyrosine Kinases-Like; TK, Tyrosine Kinases.

The well-established role of BCR-ABL1 in chronic myelogenous leukemia (CML) led us to focus our work on the inhibition of ABL1 by betulin.

2.2. Molecular mechanism of ABL1 inhibition by betulin

Following the characterization of the kinases as putative targets driving the cellular effects of betulin, we decided to explore the binding mode of betulin on ABL1 by ATP competition assays. In this study, the remaining % of maximal activity (compared with a DMSO control) was determined at ATP concentrations of 10, 50 and 100 μM. As shown in Figure 2, the results obtained strongly suggest an ATP-competitive inhibition of ABL1 by betulin. The inhibition of the ABL1 activity by 10 μM betulin was significantly decreased in the presence of a high concentration of ATP (100 μM). Note here that triterpenoids extracted from the dry infructescences of *Liquidambaris Fructus* (also called Lu Lu Tong when used in Traditional Chinese medicine to treat some breast disease) were also described as putative ATP competitors [26].

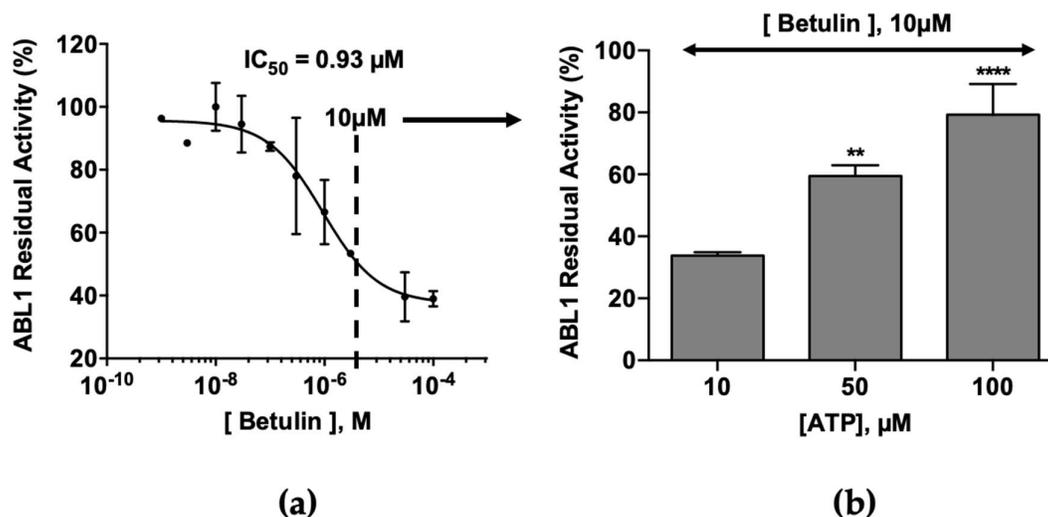


Figure 2. Effect of ATP on the inhibition of ABL1 kinase by betulin. (a) The IC₅₀ value of betulin against human ABL1 kinase was determined from the dose-response curve using GraphPad PRISM Software. ATP concentration used in the kinase assays was 10 μM (values are means, n=2). (b) We selected 10 μM of betulin as fixed concentration of inhibitor (approximately 10 times the IC₅₀ value) and measured the inhibition by betulin at different ATP concentrations by ADP-Glo luminescent assay: 10, 50 and 100 μM. Data represent the mean (n=4) ± SD expressed in % of residual activity, compared with a DMSO control. ** p<0.01 vs ATP [10 μM], **** p < 0.0001 vs ATP [10 μM].

2.3. Molecular modeling of ABL1-betulin complex

To gain further insight, we investigated the interaction of betulin with the ABL1 tyrosine kinase ATP binding site by molecular docking. For this purpose, the crystal structure of ABL tyrosine kinase complexed with imatinib (a known ABL1 inhibitor) was used, and docking was carried out with Discovery Studio 3.1 and AutoDock Vina[27,28] software. The accuracy of the docking procedure was evaluated by docking imatinib back into the binding site. The root mean square deviation (RMSD) of the highest-ranked orientation from the position of the imatinib in the crystal structure was found to be 1.01 Å (Figure 3). We note that RMSD values <1.5 Å are considered to indicate successful docking[29].

The results show that betulin fits within the ATP binding site of ABL tyrosine kinase, and overlaps with the methylpiperazine ring of imatinib (Figure 3). The positioning of betulin is similar to that predicted for a pentacyclic triterpenoid gypsogenin derivative in recent report[30]. In contrast to imatinib, the large size of the betulin molecule sterically hinders it from binding deep within the binding pocket. The secondary alcohol extends towards the exterior of the protein while the hydroxymethyl and vinyl substituents are directed towards the interior of the protein. While imatinib undergoes extensive hydrogen bonding, the lipophilic structure of betulin undergoes mostly van der Waals interactions with the surrounding amino acid residues (e.g. Glu286, Met290, Ile293, Val298, Leu354, Ile360, His361, Arg362 and Asp381).

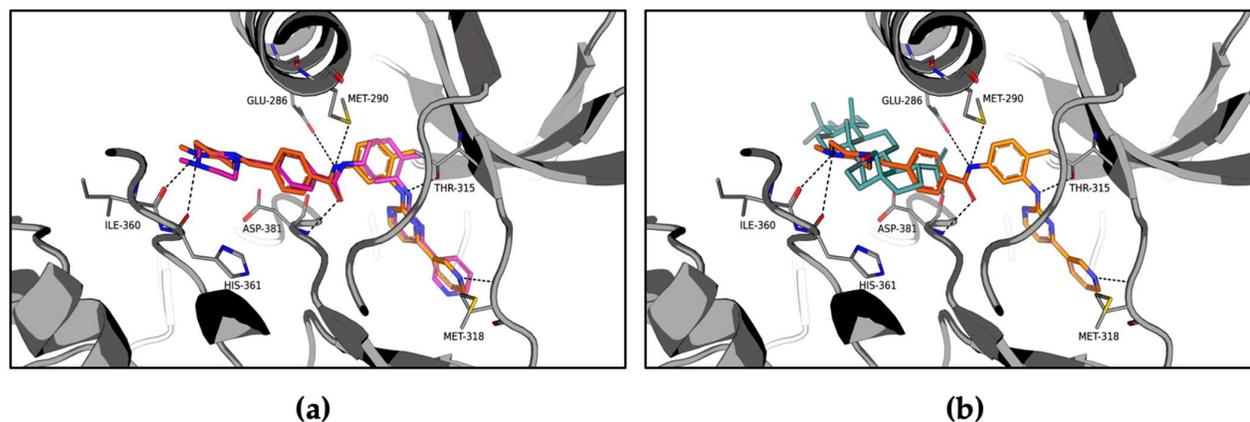


Figure 3. In silico docking analysis of the interaction between the ATP binding site of ABL1 and imatinib or betulin. (a) The binding orientation and interactions of imatinib with the ABL1 tyrosine kinase as recorded in the crystal structure (orange, PDB code: 1IEP) compared to the orientation predicted with molecular docking (magenta). (b) The binding orientations and interactions of imatinib (orange) and betulin (teal) with the ABL1 tyrosine kinase ATP binding site as predicted with molecular docking.

2.4. Betulin selectively inhibits proliferation of human leukemic cells

The anticancer and chemoprotective potential of betulin has already been reported (see [31] for a table reporting the *in vitro* antiproliferative effect of betulin on 40+ cancer cell lines). As betulin inhibited ABL1, a kinase shown to cause chronic myelogenous leukemia (CML) when deregulated by fusion with BCR, we next used the human K562 CML cell line to study the *in vitro* effect of betulin (Figure 4a). Moreover, the effect of betulin on K562 cells reported in the literature was controversial (half-maximal inhibition of cell growth, IC_{50} reported are from 14.5 μ M to >200 μ M)[31]. Quantification of cell viability was used to evaluate the bioactivity of the compound. We showed here that: (i) betulin decreased the viability of K562 leukemic cells in a 48-h assay, with an IC_{50} of 16.5 μ M; (ii) leukemia cells resistant to treatment with doxorubicin (a chemotherapeutic drug sold under the brand name Adriamycin®, see Figure S1) were equally sensitive to treatment with betulin (IC_{50} of 13.5 μ M) as doxorubicin-sensitive cells whereas they were less sensitive to imatinib mesylate (Figure 4b). Note here that effects of betulin on cell viability was not significantly inhibited by 20 μ M z-VAD-fmk, a pan-inhibitor of caspases (Figure S2).

The efficacy of cancer chemotherapy is critically dependent upon tumor cell selectivity. We next tested the effect of betulin on human peripheral blood lymphocytes (hPBLs) purified from four healthy donors. As shown in Figure 4c, treatment with betulin \leq 100 μ M does not induce a significant decrease of the viability of hPBLs cells. This result indicates an acceptable level of selectivity of betulin against cancer cells.

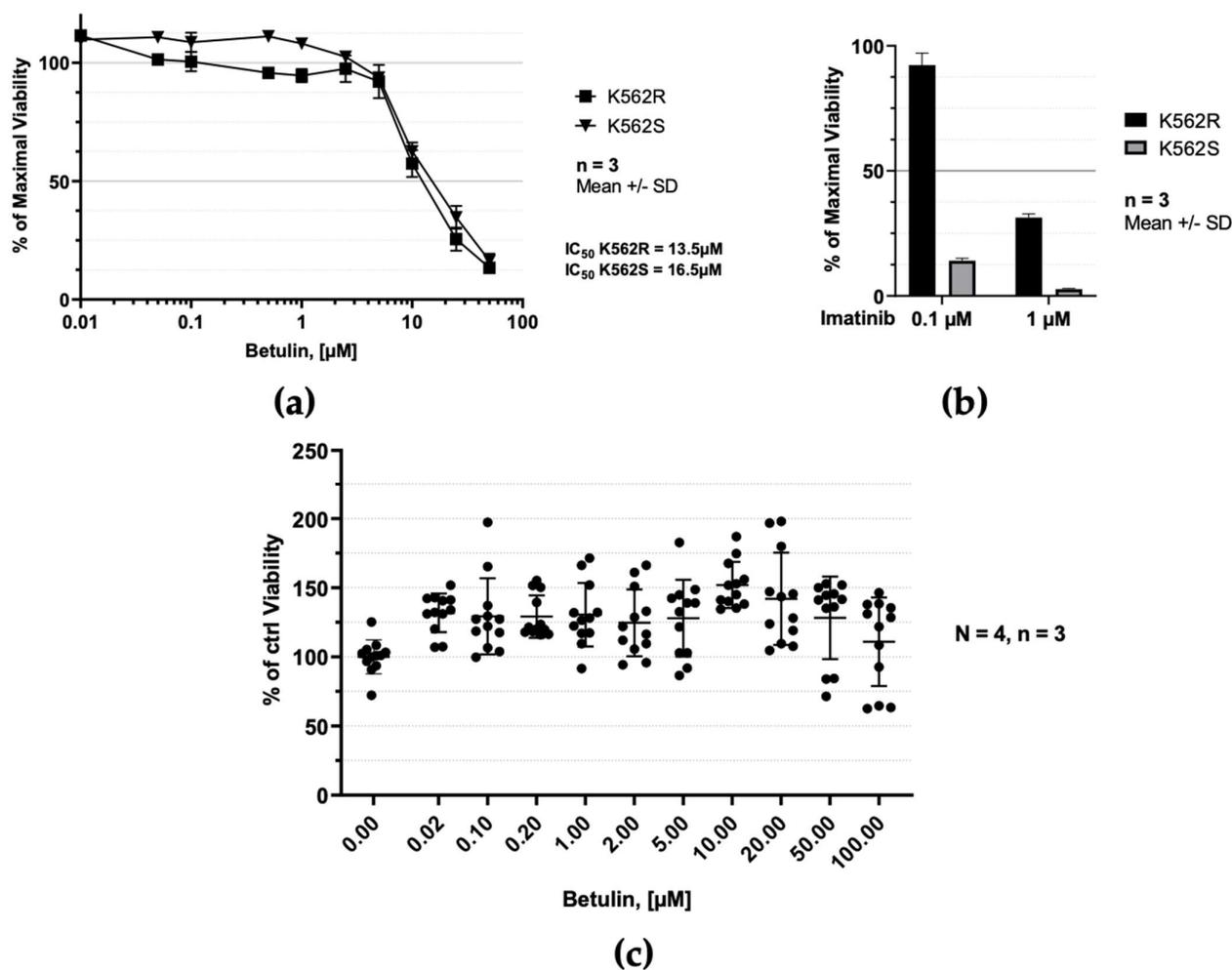


Figure 4. *In vitro* effect of betulin on the viability of K562 chronic myelogenous leukemia (CML) cells and human peripheral blood lymphocytes (hPBL). (a) The viability of K562S and K562R CML cell lines, which are respectively sensitive (S) and resistant (R) to treatment with doxorubicin, was studied by using the MTS assay. Cell viability was measured after 48-hr exposure to increasing doses of betulin. The IC_{50} values were determined from the dose-response curves using GraphPad PRISM Software. Data are given as mean \pm SD (n=3) expressed in % of maximal viability (cells treated with a similar dose of DMSO). (b) The viability of K562S and K562R CML cell lines was measured after 48-hr exposure to 0.1 or 1 μM of imatinib mesylate. Data are provided as mean \pm SD (n=3) expressed in % of maximal viability (DMSO control). (c) 48 hours after treatment with increasing concentrations of betulin (from 0.02 to 100 μM), cell viability was measured with MTS assay to evaluate the toxicity of betulin towards human PBL. Data are mean \pm SD (N=4, n=3).

2.5. Effects of Betulin on the MAPK/ERK signaling pathway

The chimeric protein BCR-ABL1 was previously shown to drive neoplastic transformation of hematopoietic stem cells in the chronic myelogenous leukemia disorder[32]. ABL1 is the kinase portion of the oncogene. When fused to BCR, ABL1 tyrosine kinase activity is constitutively activated to interact in and activate various signaling pathways including notably the mitogen activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway that leads to increased

cellular proliferation[30]. The K562 CML cell line is known to express the bcr-abl fusion gene[33]. Accordingly we examined the phosphorylation of ERK kinases in K562 (S, sensitive to doxorubicin) cells treated with 50 or 100 μ M betulin or with 20 μ M imatinib mesylate (a dose already described as effective on the ERK phosphorylation[30]). All of the treatments tested were shown to decrease the viability of K562S cells after a 48h treatment (see Figure 4a, b). In Figure 5, we extracted cellular proteins after 6h treatment, so we decided to use a higher dose of each compound compared than that necessary for efficacy on cell viability (approximately 3 and 6 times more than the IC₅₀ of betulin reported on Figure 4a). This strategy was employed to obtain a clear-cut effect on the signaling pathway. We conducted immunoblot analysis on the protein extracts using anti-phospho-ERK1/2 antibody. As shown in Figure 5 and Figure S3, betulin treatment of cells inhibited the phosphorylation of ERK in a dose-dependent manner. As a control, imatinib mesylate showed a stronger effect and almost completely abrogated the phosphorylation of ERK (Figure 5b). This result demonstrates that betulin at least partially suppresses the downstream signaling of BCR-ABL oncoprotein.

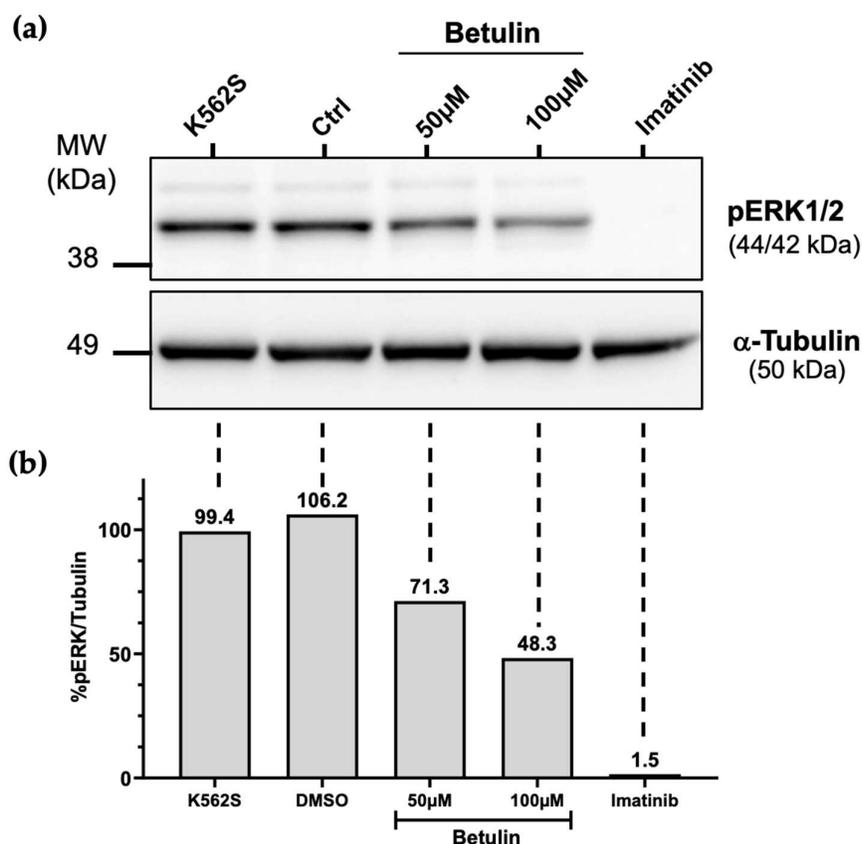


Figure 5. Effects of betulin on extracellular signal-regulated kinases (ERK) signaling. (a) K562S CML cells were untreated (K562S) or treated with 1% DMSO, 50 or 100 μ M of betulin or 20 μ M of imatinib mesylate for 6 hours and immunoblot analysis was conducted as described in the Methods section. Extracts of K562S cells were analyzed by SDS-PAGE followed by Western blotting with antibodies directed against phospho-ERK1/2 (Thr202/Tyr204) and α -Tubulin. (b) The open source image processing program "ImageJ" was used to quantify the intensity of each bands.

3. Discussion

Betulin is a naturally abundant pentacyclic triterpenoid of lupane type found in plants, especially in many species of birch trees in northern Europe. In some species the quantity of betulin can be over 50% of the dry weight of the bark[31,34]. In this study, we isolated betulin for the first time from *Acacia auriculiformis* stem bark.

Betulin and its derivatives have been intensively studied previously, exhibiting a broad spectrum of pharmacological activities, including anti-cancer, anti-viral, anti-microbial, anti-inflammatory, and anti-fibrotic effects[35,36]. Moreover, betulin-containing extracts from birch bark and formulated as an oleogel is the active pharmaceutical ingredient of Episalvan® (also known as Oleogel-S10). Oleogel-S10 was approved in 2016 by the European Medicines Agency (EMA) for treatment of partial thickness wounds in adults and since 2017 has been in a Phase III efficacy and safety study in patients with inherited epidermolysis bullosa (NCT03068780)[37]. Despite this growing interest in human therapeutics, notably for cancer treatment, the molecular mechanism of action of betulin is not well understood[31].

As previously described, triterpenoids were shown to inhibit various protein kinases: e.g. ursolic acid has been reported to exhibit anti-tyrosine kinase activity[38,39] and plant-derived pentacyclic triterpenoid gypsogenin and derivatives were reported by Ciftci *et al.* to show activity against myelogenous leukemia by virtue of their inhibition of ABL1 kinase[30]. We thus tested betulin against a panel of disease-related human kinases. Betulin was shown to inhibit several kinases from the panel tested with activity in μM range: ABL1, CK1 ϵ , GSK-3 α/β , JAK3, NEK6 and VEGFR-2.

Betulin showed notably a promising inhibitory activity against ABL1 kinase (IC₅₀ of 0.93 μM). ABL1 kinase belongs to the Abelson kinase family which includes ABL1 and ABL2. The ABL kinase family has been implicated in cancer, particularly in hematological malignancies such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and lymphoblastic leukemia[3]. At present, ABL1 kinase is among the most common drug targets of the approved therapeutic kinase inhibitors (targeted by five molecular entities approved by FDA for cancer therapy)[12,40]. The clinical success of imatinib, the first ABL1 inhibitor that reached the market in 2001, paved the way for development and use of therapeutic protein kinase inhibitors. It was shown that the activated chimeric BCR-ABL tyrosine kinase is the key biochemical defect that causes the Philadelphia chromosome-positive chronic myeloid leukemia (Ph+ CML)[40]. In our study, we showed that betulin inhibits the enzymatic activity of ABL1 and perturbs the MAPK/ERK signaling pathway in chronic myelogenous leukemia cells. Since, the antineoplastic mechanism of action of betulin is not well understood yet, our results raise the possibility that the observed antitumor effect of betulin may be at least partially explained by inhibition of kinases. We now strive to increase the panel of kinases, in order to explore more deeply the kinome and to test whether any kinases are more potently affected by betulin than those we found already.

Multidrug resistance (MDR) mediated by the drug efflux protein, P-glycoprotein (P-gp), is one of the major obstacles to successful cancer chemotherapy[41]. As an example, cancer cells use P-gp to escape cell death induced by doxorubicin chemotherapeutic agent[42]. We showed here that contrary to doxorubicin, K562 doxorubicin-resistant cells retained an undiminished sensitivity to betulin. This result indicates that betulin is probably not a substrate for the P-glycoprotein, a crucial factor in developing potent treatment strategies to combat human MDR cancer.

The results obtained in this study shed light on a putative mechanism of action of betulin that may drive its known effects on cancer cells. Indeed, Betulin, isolated for the first time from *Acacia auriculiformis*, was shown to have a multi-pharmacological profile, affecting notably ABL1, JAK3 and GSK-3 α/β . These results support the notion that betulin could be used alone or in combination with other anticancer drugs as a putative natural product-based therapeutic for the treatment of haematological malignancies disease related to deregulation of protein kinases.

4. Materials and Methods

4.1 Reagents

Betulin (Lup-20(29)-ene-3 β ,28-diol, product reference B9757, purity \geq 98%) was from Sigma-Aldrich (St. Louis, MO, USA). Imatinib mesylate (GleevecTM, product reference S1026) was from Selleckchem (Houston, TX, USA). z-VAD-fmk was obtained from Enzo Life Sciences (Villeurbanne, France). Doxorubicin was obtained from Teva Pharmaceutical (Petah Tikva, Israel). Stock solutions of chemical compounds were prepared in dimethyl sulfoxide (DMSO).

4.2 Cell lines and culture

K562 (ATCC[®], CCL-243, described here as K562S for sensitive to treatment with doxorubicin), a human chronic myelogenous leukemia cell line, was obtained from American Type Culture Collection (Manassas, VA, USA). Doxorubicin-resistant K562R (also known as K562/Adr) was kindly provided by the IRSET institute (Research Institute for Environmental and Occupational Health, INSERM, University of Rennes 1, France). The cells were maintained at 37°C and 5% CO₂ in GibcoTM 1640 Roswell Park Memorial Institute (RPMI-1640) medium containing 10% fetal bovine serum (FBS) (Life TechnologiesTM, Thermo Fisher Scientific, Waltham, MA, USA).

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from blood buffy coats of healthy donors, provided by the Etablissement Français du Sang (EFS). The research protocol was conducted under French legal guidelines. After separation of monocytes by 1 h adhesion step, non-adherent PBMCs (peripheral blood lymphocytes, PBL) were harvested. PBL were cultured in RPMI 1640 medium (Gibco, Life technologies, Carlsbad, CA, USA) supplemented with 10% decomplexed fetal bovine serum (CVFSVF00-01, Eurobio), penicillin (100 IU/ml), and

streptomycin (100 µg/ml) (15140-122, Gibco). All cells were cultured under a 5% CO₂ atmosphere at 37 °C.

4.3 Purification of natural products from plant material

Plant material (consisting of bark), were collected in Samaru-Zaria, Nigeria in September, 2018, and was identified by U.S Gallah, the plant taxonomist of Biological Sciences Department, Kaduna State University, where a voucher specimen (number 1292) was deposited in the herbarium. Relevant ethical guidelines were followed for the collect of plant material. All the protocols involving plants adhered to relevant ethical guidelines. The air-dried pulverized bark was extracted with 70% ethanol at room temperature for 7 days. The combined ethanol extract was concentrated using a rotary evaporator to give a semi-solid mass (45g). 32g of the crude extract was suspended in 100ml of water and partitioned with 5 x 300ml of ethyl acetate and 5 x 300ml of n-butanol to give 2.3g and 3.6g of ethyl acetate- and n-butanol-soluble fractions respectively. A portion of the ethyl acetate-soluble fraction (2.4g) was packed into a column of silica gel G (200-400 mesh, Silicycle, 5cm x 50cm) and eluted first with 100% dichloromethane and then with a stepwise gradient of dichloromethane and methanol mixture as follows: 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, 80:20, 60:40, 50:50, 30:70, 10:90 and 100% methanol. The progress of elution were monitored by thin layer chromatography (carried out on pre-coated silica gel TLC plates aluminum backed (Silicycle) using the solvent system Ethyl acetate:dichloromethane:methanol:water (15:8:4:1 and 6:4:4:1, respectively). Fractions eluted with 2% methanol in dichloromethane was further purified using Sephadex LH-20 (Pharmacia), eluted with methanol to give natural product-betulin, isolated as a white solid. The identification of betulin was performed by NMR spectroscopy carried out on a Bruker Avance NMR spectrophotometer (500MHz ¹H, and 125 MHz ¹³C).

4.4 Protein Kinase Assays

Kinase enzymatic activities were assayed in 384-well plates using the ADP-Glo™ assay kit following the recommendations of the manufacturer (Promega, Madison, WI). Controls were performed in appropriate dilutions of dimethyl sulfoxide (DMSO). Kinase activities, measured in the presence of 10µM ATP, are expressed as percentage of maximal activity, i.e. measured in the absence of inhibitor. In order to determine the half-maximal inhibitory concentration (IC₅₀), the assays were performed in duplicate in the absence or presence of increasing doses of the tested compounds. Data were analyzed using GraphPad PRISM (GraphPad Software, San Diego, CA, USA) software to fit a sigmoidal curve that allowed the determination the IC₅₀ values. The experimental conditions used for measuring kinase activities are comprehensively described in Ibrahim *et al.*[43].

4.5 Molecular Docking

Molecular docking simulations were carried out with Discovery Studio 3.1 (Accelrys) and AutoDock Vina software[27]. The crystal structure of ABL1 tyrosine kinase

complexed with imatinib was obtained from the Brookhaven protein data bank (PDB code: 1IEP)[28]. The protein was prepared and protonated for docking in Discovery Studio with the 'prepare protein' function. The pKa values and protonation states of the ionizable amino acids were subsequently calculated at pH 7.4, and the protein model was typed with the Momany and Rone CHARMM forcefield. A fixed atom constraint was applied to the backbone and the protein was energy minimized with the Smart Minimiser algorithm (maximum amount of steps of 50,000) using the implicit generalised Born solvation model with molecular volume. Discovery studio was used to construct the structures of betulin and imatinib, which were submitted to the 'prepare ligands' protocol. The co-crystallised ligand and water molecules were removed from the protein model, and AutoDock Vina was used for the docking. The highest ranked solution of each ligand was finally refined with the Smart Minimizer algorithm. Illustrations were prepared with the PyMOL molecular graphics system[44].

4.6 Drug treatment and Cell viability assay

Cell viability was evaluated with the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction kit, (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Fitchburg, WI, USA), according to the instructions of the manufacturer. Cell viability (% of maximal viability) was quantified following 48-hr exposure of human cells (PBLs, human K562S and K562R myelogenous leukaemia cells) to the tested doses of betulin, doxorubicin or imatinib mesylate (the latter as a positive control inhibitor of ABL1 kinase). Further details on experimental conditions used can be found in Delehouzé et al. 2017[45].

4.7 Immunoblot Analysis and Antibodies

K562S cells were treated with 20 μ M of imatinib mesylate or 50 and 100 μ M of betulin for 6 hours. The concentration of DMSO in the culture medium was 1%. Cell lysates were prepared by sonification of the cells in homogenization buffer with 0.5% of nonidet P-40 non-ionic detergent (NP-40) as described previously[46]. SDS-PAGE and immunoblotting were performed following standard procedures. Anti- α -tubulin antibody (clone B512, T5168, 1:5000) was purchased from Sigma Aldrich (St. Louis, MO, USA). The anti-phospho-p44/42 MAPK (#28733-1-AP, 1:2000) was purchased from Proteintech® (Rosemont, IL, USA). This rabbit polyclonal antibody detects p44 and p42 MAPK when dually phosphorylated (at Thr202 and Tyr204 of p44 and at Thr185 and Tyr187 of p42 MAPK).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: *In vitro* effect of doxorubicin on the cell viability of K562 chronic myelogenous leukemia (CML) cells., Figure S2: The effect of treatment with pan caspase inhibitor z-VAD-fmk on the phenotype induced by betulin, Figure S3: Effects of betulin on extracellular signal-regulated kinases (ERK) signaling.

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cell-based assays: A.A., C.D., M.S-E., M-T.D-B.; writing—review and editing, A.A.A., J.C.B., L.L., M.R., S.B.; supervision, A.A.A., S.B.; project administration, A.A.A., S.B.; funding acquisition, A.A.A., S.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Claire Delehouzé, Morgane Rousselot, Marie-Thérèse Dimanche-Boitrel and Stéphane Bach are founders and members of the scientific advisory board of SeaBeLife Biotech, which is developing novel therapies for treating liver and kidney acute disorders. This work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The other authors declare that they have no competing interests.

Sample Availability: Betulin can be purchased from Sigma-Aldrich (St. Louis, MO, USA) under the reference B9757.

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