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Original article

Synthesis and antiproliferative activity of ligerin and new fumagillin analogs against osteosarcoma

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ABSTRACT

Ligerin (**1**) is a natural chlorinated merosesquiterpenoid related to fumagillin (**2**) exhibiting a selective antiproliferative activity against osteosarcoma cell lines and an *in vivo* antitumor activity in a murine model. Semisynthesis of ligerin analogs was performed in order to study the effects of the C3-spiroepoxide substitution by a halogenated moiety together with the modulation of the C6 chain. Results showed that all derivatives exhibited an *in vitro* antiproliferative activity against osteosarcoma cell lines and that chlorohydrin compounds were equally or more active than their spiroepoxy analogs. Among semisynthetic analogs, the parent compound **1** was the best candidate for further studies since it exhibited higher or equivalent activity compared to TNP470 (**3**) against SaOS2 and MG63 human osteosarcoma cells with a four times weaker toxicity against HFF2 human fibroblasts. Quantitative video-microscopy analysis was conducted and allowed a better understanding of the mechanism of its antiproliferative activity.

1. Introduction

Despite its low incidence (around 300 new cases/year in Europe), osteosarcoma is the most frequent malignant primary bone tumor. It affects predominantly children, teenagers and young adults and accounts for 8.9% of cancer-related deaths in children [1,2]. No satisfactory treatment is currently available and no significant improvement in prognosis has been noticed since the advent of combined chemotherapy in the 90's. The 5-year survival rate for patients diagnosed with osteosarcoma is still remaining between 60% and 70% without metastasis [2]. Current therapy consists in combining surgery and multiagents chemotherapy (neoadjuvant and post-surgery) based principally on methotrexate, ifosfamide, doxorubicin and cisplatin treatments. All these drugs induce significant side effects, highlighting the need to improve current treatment strategies.

Fumagillin (**2**) is a merosesquiterpene isolated for the first time in 1949 from a crude extract of a strain of *Aspergillus fumigatus* [3,4]. First studied and used in clinical medicine for its antimicrobial activity in human and veterinary health [5], this molecule has known a renewed interest since 1990 because of its anti-angiogenic properties [6]. Given the high toxicity of this compound, many syntheses of derivatives were done and led to the synthesis of the first clinically developed analog: the 6-O-chloroacetylcarbamoyl-fumagillol named TNP470 (**3**). This compound presented a strong activity against adenocarcinoma but its clinical development was stopped in phase II trial due to its neurotoxicity [7–10]. The instability and the toxicity of TNP-470 are likely due, at least in part, to the presence of three functional groups chemically labile or metabolically unstable, the two epoxides (the spiroepoxide and the 1′-2′ epoxide on the C4 side chain) and the chloroacetyl moiety at C6.

The mechanism of action of this class of compounds remains not fully resolved, but the methionine aminopeptidase-2 (MetAP2) has been identified as their main molecular target. This metalloprotease is responsible for the removal of the methionine residue from newly synthetized polypeptides, allowing their further myristoylation and functionalization. Inhibition of this enzyme would cause cell-cycle arrest. Most of these sesquiterpenes inhibit selectively and irreversibly the MetAP2 through insertion of the C4 chain in the active pocket, mimicking the terminal part of native proteins, and via the formation of a covalent bond between the C-7 of the

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spiroepoxide group and a nitrogen of the 231-histidine residue of the enzyme [11].

In 2005, Rodeschini [12] defined the role of the two epoxides for the binding of fumagillin analogs to the active site of MetAP2 which involves two water molecules, a binuclear metal center and the 231-histidin residue [13]. As the removal of the 1′-2′ epoxide doesn't impact the activity of the molecule, it would most likely be involved in the orientation of the side chain for the recognition by the enzyme [14]. Griffith et al. defined the importance of the second epoxide, demonstrating that the removal of the ring epoxide dramatically lowered the activity of fumagillol against MetAP2 [15]. Since its first discovery and use in medicine, structural modifications on the sesquiterpene backbone concerned mainly the C4-branched chain in order to improve the affinity for the hydrophobic channel surrounding the catalytic pocket [16]. Among dozens of synthetic derivatives of fumagillin, two analogs have recently been undergoing development in phase I clinical trials. CKD-732 is studied for the treatment of refractory solid cancer [17] and in combination with capecitabine and oxaliplatine for the treatment of metastatic colorectal cancer in patients who progressed on chemotherapy based on irinotecan [18]. PPI-2458 is evaluated for the treatment of non-Hodgkin lymphomas and several solid tumors [19]. These two compounds corroborate the interest of this chemical family in the search for new anticancer drugs.

In the course of our search for new drugs against osteosarcoma, ligerin (**1**), a new natural compound related to fumagillin (**2**), has previously been isolated from a marine-derived strain of *Penicillium* sp. (Fig. 1). Ligerin, the 3-hydroxy, 3-chloromethylene, 6-(3-carboxy-1-oxopropyl)-fumagillol was the second natural product exhibiting a halogenated moiety in place of the common spiroepoxide. Assayed against various murine cell lines, it exhibited a selective antiproliferative activity against osteosarcoma compared to non-tumor cells [20].

This work was focused on the semisynthesis of new halogenohydrin analogs related to ligerin, in order to investigate the impact of halogen atoms such as chlorine or bromine on the antiproliferative activity compared to their spiroepoxide analogs. Structural modulations of C6-side chain were also explored, maintaining the terminal carboxylic acid moiety. Bioactivities as well as selectivity of all compounds were evaluated using *in vitro* assays against both murine and human osteosarcoma and non-tumor cell lines and were compared to TNP470 (**3**) and reference anticancer compounds. Further studies on the ligerin bioactivity were also conducted, in order to get a better understanding of its antiproliferative mechanism.

2. Results and discussions

2.1. Chemistry

In order to evaluate the effect of the spiroepoxide opening and its substitution by a halogenomethylene moiety on the cytotoxicity of this chemical series, 6-O-succinylfumagillol (**1a**) and fumagillol (**4a**) were synthesized together with their respective chlorohydrin analogs, ligerin (**1**) and 7-chloro-fumagillol (**4**) (Scheme 1). Given that only two brominated compounds have been described in the literature in the fumagillin series [21], the synthesis of a bromohydrin analog of ligerin was also performed (**5**). In a second time, some ligerin analogs with different C6 moieties were synthesized, by introducing a heteroatom or a benzene ring in the side chain or by extending the length of the carboxylic acid side chain.

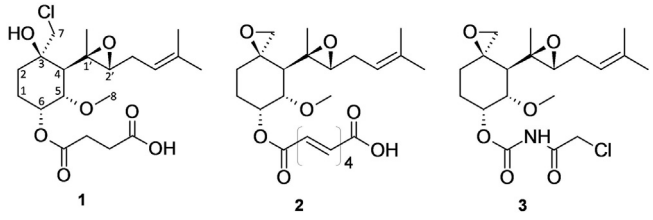

Fig. 1. Structures of ligerin (**1**), fumagillin (**2**) and TNP470 (**3**).

For that purpose, a first step consisted in preparing **4a**, the saponification product of (+)-fumagillin. Instead of the classical two steps process consisting in a first purification of the fumagillin dicyclohexylamine salt contained in a commercial product (Fumidil B®) followed by the hydrolysis of the C-6 ester, a one-step reaction was developed. In this way, the entire commercial preparation was directly submitted to alkaline hydrolysis using 0.5 N NaOH, allowing to purify **4a** from the reaction mix by liquid/liquid partition after acidification. From **4a**, each structural modulation was obtained with the same semisynthetic approach. Compound **4a** was esterified using different anhydrides to afford compounds **1a**, **6a**, **7a**, **8a**. To obtain the chlorohydrin or bromohydrin analogs (compounds **1**, **5**–**8**), a halogenation step was further performed using the corresponding halogenous salt, i.e. LiCl or LiBr. The structures of all compounds were confirmed by ^{1}H and ^{13}C NMR analyses.

2.2. Pharmacology

2.2.1. Antiproliferative activity

The antiproliferative activity of halogenated compounds (**1**, **4** and **5**) and their respective spiroepoxide analogs (**1a**, **4a**) was tested *in vitro* against two osteosarcoma cell lines: one murine (POS-1) and one human (SaOS2).

As shown in Fig. 2, compounds **1a** and **1** were more active than **4a** and **4** as no IC_{50} could be measured in the range of 0.40–2100 nM for these last compounds. This first result confirms that a C6 side-chain is required for a high activity against these cancer cells [22]. The C7 halogen substitution was shown to have a weaker influence on the cytotoxicity. The activity of the chlorohydrins and their respective spiroepoxide analogs was found to be equivalent except for **1** against SaOS2 cell line which was more active than its spiroepoxide analog **1a** and in the same manner for **4** and **4a** against POS-1 cells. Similar activity was also obtained for **3** and its halogenohydrin derivative against the two osteosarcoma cell lines. Nonetheless, substitution of the chlorine atom by a bromine atom decreased the activity as **5** exhibited an intermediate activity compared to **1** and other analogs. IC_{50} of **5** against POS-1 and SaOS2 cells were respectively 272 nM and 801 nM whereas IC_{50} of **1** were 78 nM and 137 nM.

In literature, the two epoxides have often been described as essential for the binding to MetAP2, the target enzyme of this class of compounds [12], but the impact of the C3 epoxide opening on the activity remains unclear. In this way, it has been reported that compounds for which the spiroepoxide was opened were less active than their intact analog. For instance, the C3-methylthiomethyl derivative of **3** was over 100 times less active than the parent compound on both MetAP2 inhibition and HUVEC assays [23], and halogenation of the spiroepoxide of fumagillin derivatives led to a 10 fold decrease of their activity on the enzyme [24]. On the contrary, Hayashi et al. have shown that chlovalicin, a natural chlorinated analog in the fumagillol series, was 3 times more active than its epoxidized form ovalicin [25]. More recently, in a *in vitro* metabolization study, Arico-Muendel et al. have shown that, after exposure of the fumagillin analog PPI-2458 to an acidic treatment mimicking the stomach milieu, some metabolites formed were chlorohydrin analogs. These compounds were found as effective as their epoxide precursor on a MetAP2 inhibition assay