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Synthesis and cytotoxicity evaluation of olivacine-indole hybrids tethered by alkyl linkers

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ABSTRACT

In this work, eleven new derivatives were prepared of the alkaloid olivacine (1), which was isolated from the bark of *Aspidosperma australe*. These compounds (**7a–k**) are hybrids of olivacine and indoles or carbazole, tethered by alkyl chains of variable lengths (C-4, C-5 or C-6). Compounds **7a–k** showed increased cytotoxicity towards a panel of four cell lines. The subcellular localization of olivacine and of the synthetic derivatives was studied by fluorescence microscopy. The cycles of K562 cells exposed to olivacine or compounds **7a–k** were analysed by flow cytometry, and showed, for some of the new derivatives, a different profile of cell distribution among the phases of the cycle when compared to olivacine, which is indicative of lysosomal apoptosis.

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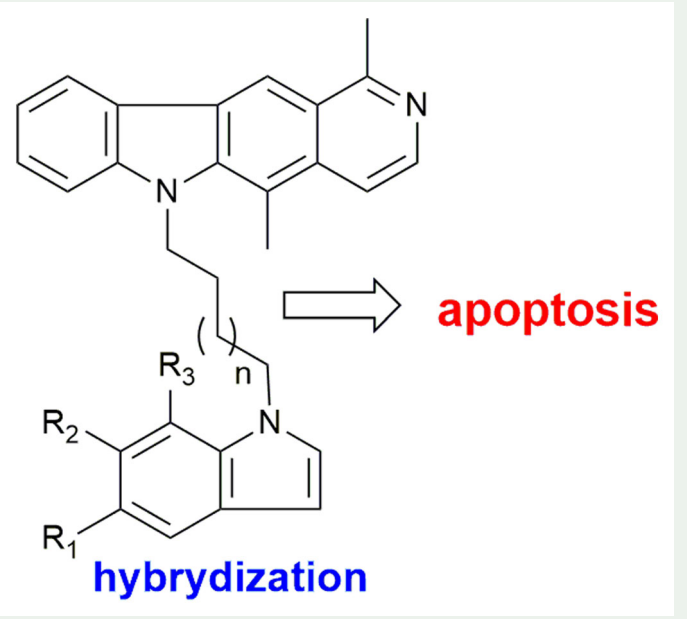

1. Introduction

In the course of a collaborative project aimed at the discovery and sustainable use of natural resources of the Atlantic rainforest of Misiones province in north-eastern Argentina, the bark tissues of several trees of this region were investigated, in the search for abundant and easy to isolate secondary metabolites that may be amenable for chemical diversification. Olivacine (**1**) was isolated as one of the main alkaloids of the bark of the "yellow guatambu" tree (*Aspidosperma australe*) together with uleine (**3**), *N*-methyltetrahydroellipticine (**4**) and apparicine (**5**) (Pérez et al. 2019) (Figure 1). From this biological source, and due to its simple purification protocol (see Supplementary Material), a steady and sustainable supply of olivacine became available (typically 0.8 g/1 kg of bark) for a chemical diversification project, with the aim to explore structural modifications that had not been thoroughly investigated in previous works.

Alkaloids with a pyridocarbazole nucleus have been intensely studied due to their planar, highly conjugated structures, and their reported antitumor activity (Asche and Demeunynck 2007; Roesch 2016; Tsutsumi et al. 2016). Olivacine (**1**), and its isomer ellipticine (**2**), are the best known alkaloids of this family. Since the first reports of their antitumor activity, these compounds have been the subject of numerous studies focused on their biological activity, modes of action and structure-activity relationships (ÓSullivan et al. 2013). Initially, ellipticine and olivacine were regarded as DNA intercalators and inhibitors of topoisomerase II (Garbett and Graves 2004). Later studies showed that these alkaloids, as well as some synthetic derivatives, have a multimodal

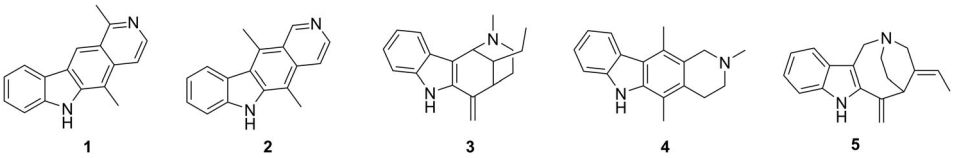

Figure 1. Olivacine (**1**), ellipticine (**2**) and other alkaloids isolated from the bark of *Aspidosperma australe*.

type of action. This includes the interaction with several proteins of the cell-cycle control system, such as p53, p21, p73 and several kinases such as AKT and c-Kit kinase (Shi et al. 1998; Jin et al. 2004; Vendôme et al. 2005). The development of several synthetic routes to olivacine triggered the preparation of a series of derivatives (Tylińska et al. 2018; Schmidt et al. 2018; (see Table S1 for a selection of structural types and additional references in Supplementary Material). In particular, one of these derivatives, S-16020-2 (1-diethylaminoethylolivacine), reached Phase II clinical trials as an anticancer drug (Léonce et al. 1996; additional references in Supplementary Material). However, olivacine still offers several unexplored possibilities of structural diversification, such as the chemistry of the indole nitrogen.

In this work, eleven olivacine derivatives (compounds **7a–k**) were prepared, which are hybrid compounds based on olivacine and a series of simpler substituted indole or carbazole structures, tethered by hydrocarbon chains of variable lengths. The rationale for the design of these hybrids was that the presence of a second planar heteroaromatic moiety connected by a linker may modify the intercalating capacity of the compounds. Several substituted indoles, as well as carbazole, were selected as the second heterocyclic moiety. All the new compounds were tested for cytotoxic activity against a panel of four cell lines, and their effect on the cell cycle was also analysed by flow cytometry. In addition, due to the intrinsic fluorescence of olivacine and compounds **7a–k**, their subcellular location was also evaluated. The derivatives of olivacine with the three *N*-bromoalkyl chains (**8a-c**) were also prepared in order to compare their bioactivity profiles with those of the hybrid compounds.

2. Results and discussion

2.1. Chemistry

Initial attempts to obtain compounds with two units of olivacine tethered by *N*-alkyl chains by typical S_N2 reactions of the alkaloid with the corresponding alkyl dihalides and base, gave only the monosubstituted *N*-alkylated product, in which a bromine substituent was still present at the end of the alkyl chain. Although different conditions were tried for this transformation (use of different solvents, bases, temperatures, microwave heating, ionic liquids), the steric hindrance of the olivacine core (especially C5-Me) prevented the formation of the disubstituted compounds in reasonable yields. However, in a previous work with simpler indoles, geminal disubstituted compounds could be readily prepared from alkyl dihalides and an excess of indole under the same conditions (Bertinetti et al. 2011). In view of this, we decided to prepare hybrid compounds with an olivacine core and simpler, sterically less challenged indoles, by two consecutive S_N2 reactions as outlined in Figure 2.

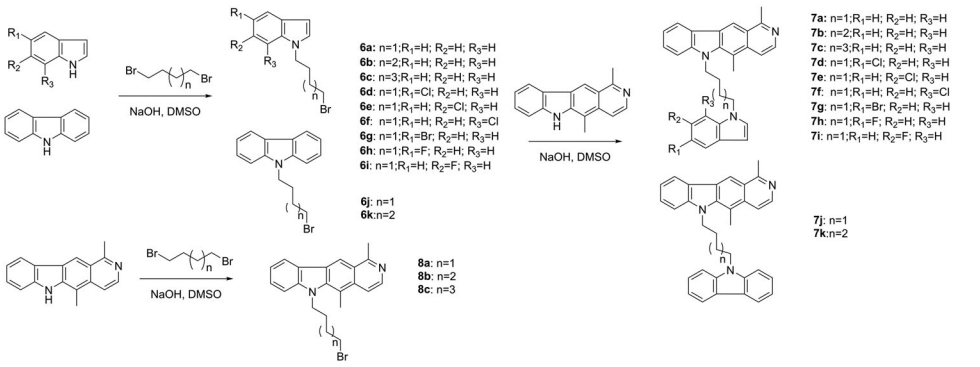

Figure 2. Synthesis of compounds **6a-k**, **7a-k** and **8a-c**.

Although the order of the two S_N2 *N*-alkylations could be interchanged with comparable yields, a conservative approach was adopted regarding the natural product component, trying to minimize the purification steps of the olivacine-containing products. In this way, different substituted indoles, as well as carbazole, were first treated with an excess of NaOH in DMSO, and after the formation of the corresponding anions, *N*-alkylation with the alkyl dihalide (1,4-dibromobutane, 1,5-dibromopentane or 1,6-dibromohexane) yielded the *N*-bromoalkylindoles **6a–k**. These intermediates, after quick purification by flash column chromatography, were used as electrophyles in a second S_N2 step, this time with the anion of olivacine in DMSO as nucleophyle, to yield the final hybrids **7a–k**. Full experimental procedures can be found in the Supplementary Material. Derivatives of olivacine with the *N*-bromoalkyl chains (**8a-c**) were also prepared by the same technique, in order to study the influence of the tether or the second heterocyclic moiety on the bioactivity profile.

2.2. Biological assays

2.2.1. Cytotoxicity Evaluation

The cytotoxicity of olivacine and compounds **7a–k** and **8a-c** was tested against a panel of four cell lines: LM2 (murine mammary adenocarcinoma), K562 (human leukemia), IGROV-1 (human ovarian cancer) and HaCaT (human normal keratinocytes) (Van Merloo et al. 2011). Doxorubicin and cisplatin were used as positive controls. All IC_{50} values are listed in Table 1. From these results, it is clear that all the derivatives show an increase in cytotoxic activity (two or three-fold) compared to olivacine. The observed IC_{50} are in the same range as those of the clinical drugs doxorubicin and cisplatin. However, there are no marked differences in the bioactivity of the hybrids with different indole moieties or tethers of different length. There is also a lack of selectivity against the different cell lines, either normal or tumoral.

2.2.2. Fluorescence microscopy

Since olivacine and compounds **7a–k** are all fluorescent, their subcellular location was studied by fluorescence microscopy. LM2 cells grown on coverslips were analysed by fluorescence microscopy after 10 min and 48 h exposures to the compounds. A fast

Table 1. Cytotoxicity evaluation for compounds **1,7a-k** and **8a-c**.

	IC_{50} (μM)[a]			
Compound	LM2	HaCaT	K562	IGROV-1
1	11.2 ± 2.7	11.8 ± 2.5	5.9 ± 0.7	9.8 ± 1.1
7a	4.2 ± 0.5	4.8 ± 0.4	2.3 ± 0.2	2.9 ± 0.3
7 b	3.6 ± 0.4	4.1 ± 0.6	1.9 ± 0.1	2.7 ± 0.3
7c	5.0 ± 0.3	5.6 ± 0.3	2.5 ± 0.4	3.0 ± 0.4
7d	6.0 ± 0.7	7.1 ± 0.8	3.1 ± 0.5	2.6 ± 0.2
7e	3.8 ± 0.5	4.2 ± 0.5	1.7 ± 0.2	2.9 ± 0.4
7f	4.9 ± 0.3	5.0 ± 0.4	2.8 ± 0.3	3.7 ± 0.5
7 g	4.0 ± 0.5	4.5 ± 0.5	2.0 ± 0.5	3.2 ± 0.3
7 h	3.6 ± 0.4	4.1 ± 0.3	1.8 ± 0.2	3.3 ± 0.2
7i	3.2 ± 0.6	3.6 ± 0.7	1.4 ± 0.3	3.9 ± 0.7
7j	4.6 ± 0.4	5.8 ± 0.6	2.3 ± 0.2	4.5 ± 0.5
7k	5.2 ± 0.7	6.1 ± 0.7	3.5 ± 0.3	4.0 ± 0.4
8a	3.4 ± 0.5	3.8 ± 0.5	1.8 ± 0.2	2.8 ± 0.3
8 b	5.5 ± 0.4	5.9 ± 0.6	3.2 ± 0.4	5.5 ± 0.4
8c	5.3 ± 0.6	4.9 ± 0.5	2.5 ± 0.3	5.4 ± 0.2
Doxorubicin	1.12 ± 0.02	1.83 ± 0.18	0.44 ± 0.04	1.60 ± 0.02
Cisplatin	6.9 ± 0.8	10.9 ± 1.3	3.5 ± 0.6	7.8± 0.9

[a]MTT method after 48 h. incubation.

incorporation of olivacine and its derivatives was detected by the observation of an intense blue/green fluorescence. Figure S15 (Supplementary Material) shows the micrographs of LM2 cells exposed to olivacine or compound **7c** as a representative hybrid. The micrographs corresponding to the rest of the compounds are shown in Figures S16 and S17 in the Supplementary Material section. In the case of olivacine, the fluorescence is mostly blue, and is located mainly at the cytoplasmatic organules together with some weaker nuclear staining. However, in the case of the hybrids, the fluorescence is greener, and located mainly at the lysosomes with almost no fluorescence at the nuclei.

Previous studies of the fluorescence emission spectrum of the related alkaloid ellipticine showed that it is composed of two bands: a blue band (440 nm) which is favored at basic pH, characterizes the neutral form. Another intense green band (520 nm), is favoured in more acidic conditions and characterizes the protonated form (Schwaller et al. 1991). This fact suggests that a green fluorescence may be associated to a more acidic lysosomal location. This preferential lysosomal location of the hybrids at short incubation times, together with their weaker nuclear fluorescence when compared to olivacine may indicate a diminished nuclear uptake and DNA intercalation, and these facts point to a different mechanism of action. The cytotoxic action of the compounds is evidenced by the presence of a lower cell density in the micrographs obtained after an incubation of 48 h (see Supplementary Material).

2.2.3. Flow cytometry

The effect of olivacine and the synthesised hybrids on the cycle of K562 cells was evaluated by flow cytometry. The analysis of the histogram of olivacine at two different concentrations: IC_{25} and IC_{50} (Figure S18, Supplementary Material), allowed the following conclusions: at lower cytotoxic doses of olivacine, the cell cycle arrest is probably due to DNA topoisomerase failure (Sharma et al. 2012), whereas at higher doses, an apoptotic pathway is likely to take place. The cytoplasmatic (probably lysosomal) and

nuclear localization of the compound, are in accordance with this dual apoptotic and cycle arrest cell death.

Surprisingly, the histograms of some of the hybrid derivatives were strikingly different. Figure S19 (SupplementaryMaterial) shows a comparison of the cell-cycle profiles of olivacine and compounds **7a–k**. Most of the derivatives (especially **7a**, **7e-h**) show a dramatic increase in sub G1 cells, which more than doubles the corresponding percentage observed in the case of olivacine, together with a decrease of the number of cells in the G2/M phase. These results suggest that at high concentrations (IC_{75}), olivacine may be acting preferentially as an inducer of cell cycle G2/M arrest, whereas the derivatives trigger cell death mainly by apoptosis. These observations are in line with the subcellular localization pattern of the derivatives, which is mainly lysosomal even at long incubation periods, suggesting that a lysosomal apoptotic pathway is likely to be involved. However, the contribution of apoptotic death mediated by cell cycle arrest due to DNA topoisomerase failure cannot be fully discarded. It is interesting to note that in the case of compounds **8a-c**, in which olivacine has an *N*-bromoalkyl chain bound to the indole nitrogen but no second heteroaromatic ring, although their IC50 are in the same range as those of the hybrids, their cell cycle profiles are very similar to that of olivacine. These results indicate that the introduction of an alkyl-tethered additional heterocyclic moiety induced a different mode of action in derivatives **7a–k**.

3. Conclusions

Almost all of the previously reported derivatives of olivacine were obtained by total synthesis, however, in the present study, the derivatives were prepared from the natural product itself, as a way to add value to the natural resource. A literature search showed that there are no reported derivatives of olivacine with indole *N*-alkyl groups larger than a methyl group. In this sense compounds **7a–k** represent a new class of olivacine derivatives, which are nearly three times more cytotoxic than the parent compound, and, at the same time, display a different mode of action. In the case of olivacine, inspection of the subcellular location by fluorescence microscopy revealed that, although there is some fluorescence at the nuclei, most of the compound remains in the cytoplasmic organules. This dispersion of the compound at the subcellular level is in accordance to the previously reported multimodal action of olivacine. On the other hand, there is very little uptake of compounds **7a–k** into the nuclei, and consequently these compounds do not interact with DNA. The green fluorescence observed in the cytoplasm may be indicative of a lysosomal location of compounds **7a–k**, taking into account that lysosomal pH is particularly acidic. A striking difference in the mode of action of olivacine and **7a–k** is evident by comparison of the cell-cycle profiles obtained by flow cytometry. Most of these derivatives show a marked increase of sub G1 cells, which is indicative of apoptosis. All these results put together point towards a possible mechanism of lysosomal apoptosis for compounds **7a–k**, which in turn may explain the lack of selectivity of the compounds against the different cell lines. The fact that compounds **8a-c** show cell-cycle profiles comparable to olivacine proves that the hybridization strategy of tethering a second heteroaromatic moiety

causes this change in the mechanism of action. Finally, it must be noted that the structural modifications performed in this work can be easily combined with the previously reported synthetic strategies, in the search for more active olivacine derivatives.

Disclosure statement

No potential conflict of interest was reported by the authors.

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