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C-11 diamino cryptolepine derivatives NSC748392, NSC748393 and NSC748394: Anticancer profile and G-quadruplex stabilization

João Lavrado [a], Anthony P. Reszka [b], Rui Moreira [a], Stephen Neidle [b], Alexandra Paulo [a,*]

[a] iMed.UL – Faculdade de Farmácia, Universidade de Lisboa, Av Prof. Gama Pinto 1649-003 Lisboa, Portugal
[b] CRUK Biomolecular Structure Group, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, UK

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ABSTRACT

G-Quadruplex DNA ligands are promising novel anticancer agents with potentially fewer side effects and greater selectivity than standard anticancer drugs. However, the design of G-quadruplex ligands remains challenging since known chemical features increasing selectivity have often compromised drugability. Three C-11 diamino cryptolepine derivatives, with significant chemical differences between the side chains, low cytotoxicity to mammalian non-tumor cells (Vero cells) and drug-like properties, were selected for anticancer drug screening in the NCI Developmental Therapeutics Program. The three compounds showed good in vitro anticancer profiles with GI_{50} averages at sub-micromolar concentrations (0.32–0.78 μM), cytostatic effects (TGI) at micromolar concentrations (1.3–6.9 μM) and moderate cytotoxic effects to cancer cells (LC_{50}) also at micromolar concentrations (4.7–33 μM), but only the compound with a linear alkylamine side chain (NSC748393) showed a good score in the in vivo anticancer Hollow Fiber assay. COMPARE analysis of growth inhibition profile of NSC748393 suggested a multi-target mechanism. G-Quadruplex DNA binding affinity and selectivity studies by FRET-melting assays showed that NSC748392 and NSC478393, with aliphatic amine side chains, are good G-quadruplex ligands but not selective, whereas a C-11 aromatic side chain, as in NSC748394, increases selectivity although with decreasing binding affinity. Overall, NSC748393 can be considered a lead molecule for the design of effective but more selective anticancer drugs targeting telomeric G-quadruplexes.

Nature has contributed over the years to the development of new drugs and natural products possessing broad chemical diversity by providing clinically important anticancer drugs such as taxol, rapamycin, vincristine and epothilones A and B.[1] Natural indoloquinoline alkaloids also display in vitro cytotoxic activity against several tumor cell lines, particularly those having a methyl group in the quinoline nitrogen, such as cryptolepine (5-methyl-*5H*-indolo[3,2-*b*]quinoline).[2–4]

Cryptolepine (Fig. 1) shows broad-spectrum of biological activity, which include antibacterial, antifungal, antihyperglycemic, antimalarial, anticancer, and several others.[5] Lisgarten et al. showed that cryptolepine binds to DNA through intercalation with CG-rich sequences containing non-alternating CC sites.[6,7] This interaction with DNA is probably responsible for the cytotoxic effects of cryptolepine through inhibition of DNA synthesis and interfering with topoisomerase II in cells.[3,8,9]

Over the past few years, several indolo[3,2-*b*]quinoline analogues have been designed and synthesized as anticancer agents[10–17] particularly as telomerase inhibitors,[18–23] an emerging selective anticancer target. Erosion of telomeres, which consist of repeated guanine(G)-rich DNA sequences, plays a key role in restricting proliferation of human cells, but in most cancer cells telomerase, the enzyme responsible for

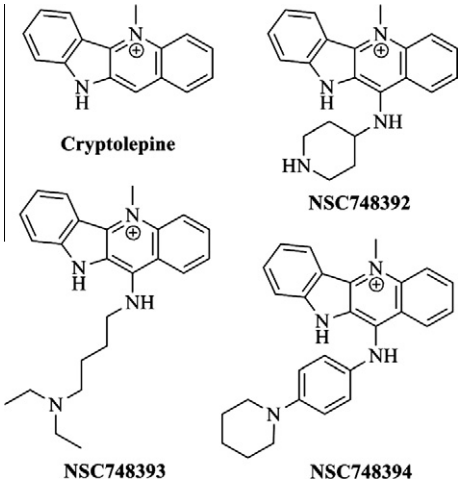

Fig. 1. Structure of cryptolepine and derivatives NSC748392, NSC748393 and NSC748394.

* Corresponding author. Tel.: +351 217 946 400; fax: +351 217 946 470.
E-mail address: mapaulo@ff.ul.pt (A. Paulo).

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Table 1
In vitro anticancer activity of NSC748392, NSC748393 and NSC748394 in the NCI human 60 cancer cell line panel. Growth Inhibition (GI_{50}), Total Growth Inhibition (TGI) and Lethal half-Concentrations (LC_{50}) are expressed in Molar units.

	NSC748392			NSC748393			NSC748394		
	Log GI_{50}	Log TGI	Log LC_{50}	Log GI_{50}	Log TGI	Log LC_{50}	Log GI_{50}	Log TGI	Log LC_{50}
Average	−6.11	−5.16	−4.31	−6.3	−5.47	−4.48	−6.49	−5.89	−5.33
Delta	1.42	1.34	1.17	0.98	1.16	1.75	0.98	1.16	1.75
Range	3.53	2.5	1.48	3.28	2.63	2.23	1.73	2.47	2.16

maintaining telomeric DNA length, is over-expressed, leading to cellular immortalization.[24]A strategy to impair telomerase activity in cancer cells is to induce the linear G-rich sequence of telomeres to form intramolecular G-quadruplex structures, which can no longer be recognized by the telomerase RNA template.[25] Cryptolepine itself shows weak telomerase inhibition activity, probably reflecting the preference of cryptolepine for a duplex rather than a G-quadruplex DNA structure.[26] Recently, a small series of cryptolepine derivatives was synthesized as telomeric quadruplex ligands and telomerase inhibitors. It was shown that the cryptolepine derivative bearing at C11 a 3-diethylaminopropylamino side chain binds preferentially to DNA-quadruplex over DNA-duplex structures and long-term exposure of cancer cells HL-60 to the 7-fluoride analogue resulted in the cellular senescence phenotype and shortening of telomere length, suggesting that C-11 alkylamine cryptolepine derivatives are promising selective anticancer agents.[27]

We have synthesized a series of C-11 alkyl, cycloalkyl and arylamine derivatives of cryptolepine with the aim of further investigating the anticancer potential and G-quadruplex stabilizing capability of this category of compounds. They have been evaluated for cytotoxicity to mammalian non-tumor cells (*Vero* cells) and drugability properties (Supplementary data), in order to select three drug-like and representative compounds of the series. We report here the in vitro and in vivo anti-cancer profiles evaluated by the National Cancer Institute Developmental Therapeutics Program (NCI-DTP), as well as the stabilization of a human telomeric G-quadruplex structure induced by the three least cytotoxic compounds of each group of analogues (incorporating alkyl, cycloalkyl or aryl side chains) and also presenting drug-like properties. Structures of the selected compounds (NSC748392, NSC748393 and NSC748394) are presented in Figure 1.

Cryptolepine derivatives NSC748392, NSC748393 and NSC7483 94 were synthesized, via 5-methyl-11-cloro-indolo[3,2-*b*]quinolinium chloride intermediates, based on the procedure developed by Görlitzer and Weber[28,29], adapted by Bierer[30,31] and described elsewhere.[32]

The lack of anticancer selectivity shown by cryptolepine and its derivatives have been associated with their binding affinity to DNA duplex structures and consequent general cytotoxicity.[27] The three cryptolepine C11-substituted derivatives selected to interrogate the NCI60 anticancer drug screening program[33], NSC748392, NSC748393 and NSC748394, represent the three types of C11-side chains studied here: cycloalkyl, alkyl and aryl, respectively. Additionally they showed low cytotoxicity to mammalian non-tumor cells (IC_{50} = 5, 10 and 62 μM) comparable to that shown by standard anticancer drug doxorubicin, 6 μM, as well as good drug-like properties according to the "rule of five": Log $P \leqslant 5$, M.W. $\leqslant$ 500 Da, H bond acceptors $\leqslant$ 10 and H bond donors $\leqslant 5$[35] (see Supplementary data for detailed data).

The NCI in vitro anticancer screen utilizes 60 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney.[36] The aim is to prioritize for further evaluation compounds showing selective growth inhibition or cell killing of particular tumor cell lines. The screening is a two-stage process, beginning with the evaluation of all compounds against the 60 cell lines at a single dose of 10 μM. All cryptolepine C11-diamine derivatives tested exhibited significant growth inhibition and so were approved for the second-stage process consisting of evaluation of compounds against the 60 cell-line panel at five concentration levels. Three dose-response parameters were calculated for each compound and each cell line: (i) drug concentration resulting in a 50% cell growth inhibition (GI_{50}), (ii) drug concentration resulting in total growth inhibition (TGI), which represents the drug cytostatic effect and (iii) concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC_{50}), representing the cytotoxic effect. Results are summarized in Table 1 and complete information on cancer cell lines responses given in the Supplementary data. All compounds had average GI_{50} responses at sub-micromolar concentrations (0.32–0.78 μM), cytostatic effects at micromolar concentrations (averages ranging from 1.3 to 6.9 μM) and also average cytotoxic effects on cancer cells at micromolar concentrations (4.7–33 μM). Moreover, compounds showed a high degree of variability in their responses, particularly those with alkyl and cycloalkyl side chains (NSC 748392-3) which showed a greater than threefold range of Log GI_{50} values. These ranges indicate selective growth inhibition of some cancer cell lines and suggest a correlation between activity and a particular mechanism of action. Additionally, the Mean Graphs of cancer cell line responses to NSC 748392 and NSC 748393 (Supplementary data) indicate that growth of most non-small cell lung cancer cells is particular inhibited by these two compounds while the largest cytotoxic effects are observed for melanoma cell lines. This may suggest different mechanisms of growth inhibition and cytotoxicity. Delta values, calculated as the difference between the average of a dose-response parameter (Log GI_{50}, Log TGI or Log LC_{50}) and the best dose-response for that parameter, range from 0.6 to 1.75 (Table 1). The highest delta value (1.75) was found for cytotoxicity (Log LC_{50}) of NSC 748393 against the melanoma cell line UACC-62, indicating that this compound is 56-fold more active in this cell line than the average cytotoxic responses in the other 60 cell lines. Based on these results, particularly on the finding of selective growth inhibition, the three compounds have been approved by NCI-DTP for in vivo antitumor efficacy tests.

By using the COMPARE program[33,34] it is possible to assign a putative mechanism of action to a test compound by determining that the response pattern is similar to that of any of the NCI database standard prototype compounds, which include alkylating agents, antimitotic agents, topoisomerase I inhibitors, topoisomerase II inhibitors, RNA/DNA antimetabolites and DNA antimetabolites.[36] The 60 cancer cell growth responses to alkyl derivatives NSC748392-3 showed a pattern similar to that of known anticancer agents, particularly to bisantrene hydrochloride, with PCC of 0.675 and 0.699, respectively. This anticancer agent binds to DNA, inhibiting topoisomerase II and consequently blocking DNA replication and cell division. However, the pattern of cancer cell growth responses to NSC748393 is also similar to that of the antimitotic vinblastine (PCC = 0.681). Additionally, the growth inhibition pattern of aryl derivative NSC748394 is not similar (PCC <6.0) to any of the 171 standard anticancer compounds present

Table 2
Acute toxicity and in vivo anticancer efficacy of NSC748392, NSC748393 and NSC748394 in the Hollow Fiber assay.

	Compounds		
	NSC748392	NSC748393	NSC748394
MTD (mg/Kg/dose)	25.0	50.0	160.0
High dose	9.4 mg/Kg	20 mg/Kg	60–30 mg/Kg
IP score	0 out of 48	30 out of 48	2 out of 48
SC score	2 out of 48	0 out of 48	0 out of 48
Total score	2	30	2
Cell kill	No	No	No

Table 3
Thermal stabilization (ΔT_m) data for cryptolepine derivatives, with a human telomeric G-quadruplex and a duplex DNA.

Compound (1 μM)	G-Quadruplex DNA (Q) ΔT_m (°C)	Duplex DNA (D) ΔT_m (°C)	Selectivity *Q*/*D*
NSC748392	19.9	15.1	1.3
NSC748393	20.9	13.1	1.6
NSC748394	4.2	1.2	4

Esds are ±0.1 °C.

in the NCI database. Taken together, the low PCC values obtained suggest that C11-diamine cryptolepine derivatives may have a mechanism of action different from standard anticancer drugs or act by more than one mechanism.

Acute toxicities of compounds were evaluated by determining the maximum tolerated doses (MTD).[37] Cycloalkyl derivative NSC748392 showed to be the most toxic with a MTD of 25 mg/Kg/dose and aryl derivative NSC748394 the least toxic one (Table 2). MTD are then used to calculate the amount of material administered to mice during anti-tumor testing. In vivo efficacy of compounds was evaluated by the Hollow Fiber Assay.[38] This assay provides quantitative indices of drug efficacy and is currently being utilized as the initial in vivo screen for agents found to have reproducible activity in the in vitro anticancer drug screen.[38] Compounds showed in vivo anticancer efficacy at zero toxic concentrations (Table 2), particularly NSC748393 is as active as current anticancer agents (total score >20) in a broad range of tumor cell lines, including melanoma, breast, colon, ovarian, non-small cell lung and CNS cancer.

G-quadruplex DNA stabilization of cryptolepine derivatives were assessed with a FRET-melting assay[39] using a 21-base oligonucleotide (F21T), that mimics the human telomeric repeats containing four G-tracts, which enables the thermal denaturation profile of the folded oligonucleotide to be characterized.[40] Thermal stabilization (ΔT_m) data (Table 3) shows that the C-11 diamino alkyl cryptolepine derivatives are effective G-quadruplex DNA ligands, with ΔT_m values of 21 and 20 °C for NSC748393 and NSC748392, respectively. These results also show that there are no significant differences in G-quadruplex binding between a compound with a linear alkyl tertiary amine side chain (NSC748393) and one with a conformationally-restricted cycloalkyl side chain bearing a secondary amine (NSC748392). However, the introduction of an aromatic amine side chain at the C-11 position of the cryptolepine scaffold (NSC748394) drastically decreases G-quadruplex stabilization (ΔT_m = 4 °C), probably reflecting both the large decrease in basicity of the side-chain aromatic amine and the decrease in net positive charge of the cryptolepine N5-methyl group due to the electronic-withdrawing effect of the C-11 aromatic side chain. It is also apparent that G-quadruplex binding of compounds with C-11 diamino linear alkyl side chains comprising a $-(CH_2)_3-$ linker (ΔT_m = 19 °C for 2d in Ref. 27) and a $-(CH_2)_4-$ linker (NSC748393), is similar.

Despite the high stabilization of G-quadruplex DNA structures by cryptolepine derivatives with aliphatic amine side chains, selectivity was observed to be low (*Q*/*D* <2) when compared with binding to duplex DNA (Table 3). It is notable that a C-11 aromatic side chain increases selectivity (*Q*/*D* = 4) albeit with decreasing binding affinity.

In conclusion, telomeric G-quadruplex structures are promising targets for the design of compounds with high cancer selectivity and low toxicity than standard DNA targeting anticancer drugs. Cryptolepine derivatives with C-11 linear alkylamine side chains were previously shown to be good G-quadruplex stabilizers, telomerase inhibitors and able to cause senescence to cancer cells after long term exposure to sub-toxic concentrations.[27] Herein we described the in vitro and in vivo anticancer profile and G-quadruplex stabilization capacity of three cryptolepine derivatives bearing significant chemical differences at C-11 amine side chains.

All three compounds NSC748392, NSC748393 and NSC748394 showed good in vitro anticancer profile in the NCI60 anticancer drug screening, but no single putative mechanism of action could be assigned using the COMPARE analysis, suggesting a different mechanism from standard anticancer drugs, or possibly a multi-target mechanism. We found when analysing the G-quadruplex DNA stabilizing capacity of these compounds, that NSC748392 and NSC478393 with aliphatic amine side chains are potent G-quadruplex ligands but are not selective for quadruplex DNA, which can explain the similar half-concentration values (in the micromolar range) found for cytotoxicity of compounds against *Vero* cells and cancer cells. Moreover, binding affinity to DNA correlates directly with basicity of side chain terminal amine, in agreement with a number of previous reports (see e.g., Refs. 41–43), whereas an aromatic amine side chain increases selectivity to G-quadruplex DNA structures.

These results, in particular the observations of selective in vitro growth inhibitory activity in cancer cells and the large G-quadruplex DNA stabilization effects, suggest that cryptolepine derivatives containing aliphatic amine side chains are potential lead molecules for the design of effective compounds targeting telomeric G-quadruplexes providing selectivity is improved.[44] It is unsurprising that the present compounds are not selective, given their mono-substitution. More complex substitution is likely to enhance selectivity, as has been found in other series.[41–43] Further studies will examine their ability to inhibit telomerase and induce senescence in susceptible cancer cell lines.

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Supplementary data

Supplementary data (physicochemical properties and cytotoxicities to mammalian non-tumor cells (IC_{50}) of cryptolepine analogues as well as mean graphs of NCI 60 cell 5-dose screen) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.110.

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