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Synthesis of cryptolepine analogues as potential bioreducible anticancer agents

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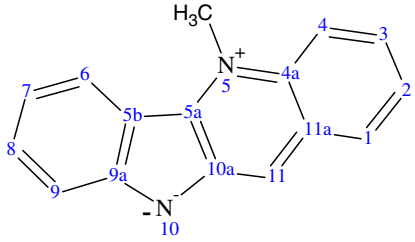
Abstract—A series of 10 novel nitro-analogues of cryptolepine (**1**) has been synthesised and these compounds were evaluated for their in-vitro cytotoxic properties as well as their potential for reductive activation by the cytosolic reductase enzymes NQO1 and NQO2. Molecular modelling studies suggest that cryptolepine is able to fit into the active site of NQO2 and thus raising the possibility that nitro-analogues of **1** could act as bioreductive prodrugs and be selectively reduced by NQO1 and NQO2 to more toxic species in cancer cells in which these enzymes are over-expressed. Analogues were screened against the RT112 cell line (high in NQO2), in the presence and absence of the essential cofactor dihydronicotinamide riboside (NRH), whereby all analogues were shown to be cytotoxic (IC_{50} < 2 μM) in the absence of NRH. With the addition of NRH, one analogue, 2-fluoro-7,9-dinitrocryptolepine (**7**), exhibited a 2.4-fold increase in cytotoxic activity. Several nitro-derivatives were also evaluated as substrates for purified human NQO1 and analogues that were found to be substrates were subsequently tested against the H460 (high NQO1) and BE (low NQO1) cell lines to detect in-vitro activation by NQO1. The analogue 8-chloro-9-nitrocryptolepine (**9**) was found to be the best substrate for NQO1 but it was not more toxic to H460 than to BE cells. Fluorescence laser confocal microscopy of **1** and several analogues showed that in contrast to **1** the analogues were not localised into the nucleus suggesting that their cytotoxic mode(s) of action are different. This study has identified novel substrates for both NQO1 and NQO2 and further work on nitrocryptolepine derivatives as a lead towards novel anticancer agents would be worthwhile.

1. Introduction

Cryptolepine (**1**, 5-methyl-10*H*-indolo[3,2-*b*]quinoline) is an indoloquinoline alkaloid which was first isolated from the roots of *Cryptolepis triangularis*[1] and later isolated as the major constituent in the roots of the West African climbing shrub *Cryptolepis sanguinolenta* (family Periplocaceae)[2] which is traditionally used in the form of a decoction in Central and West Africa, essentially for the treatment of malaria,[3] as well as for a number of other infectious and non-infectious diseases.[4]

To date, the major focus in our laboratory has been the development of new antimalarial agents and several synthetic analogues of **1** have been shown to have potent antiplasmodial activities in vitro as well as promising in-vivo antimalarial activities.[2,5] Previous work has also



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shown **1** to be a potential anticancer agent[6] due to its ability to intercalate into DNA, preferentially at GC rich sequences and non-alternating CC sites[7] with inhibition of topoisomerase II as well as DNA synthesis.[8] However, anticancer agents that target DNA are generally relatively non-selective since normal cells as well as tumour cells are affected by the drugs resulting in toxicity. A possible solution to this problem is the development of bioreducible prodrugs that would be bioreductively activated by enzymes present in tumour cells at elevated levels in comparison to normal cells.[9] One such enzyme is NQO2 (NRH:quinone oxidoreduc-

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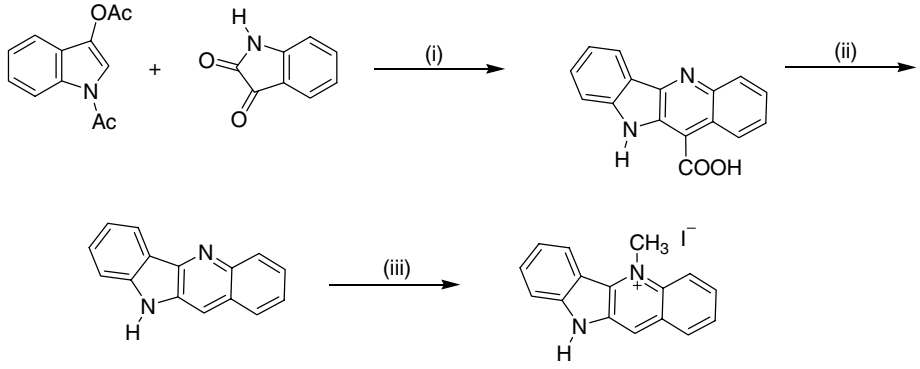
tase 2) and computational studies have shown that cryptolepine is one of only a few structural types able to fit into the active site of NQO2 (Jenkins and Moores, unpublished data, personal communication). This strategy is supported by the previous development of the dinitrobenzamide compound CB 1954 which is selectively reduced by NQO2, to a more toxic hydroxyl amino derivative which is then acetylated in vivo by acetyl-coenzymes to form a species that can form DNA inter-strand crosslinks.[10] This prodrug is currently in phase I clinical trials by Cancer Research UK.

In this study, we report the synthesis of a series of nitrocryptolepine analogues and their evaluation as bioreducible cytotoxic agents by comparing their activities in H460 and BE cell lines that express high or low amounts of NQO1, respectively,[12] and in a cell line (RT112) expressing high levels of NQO2 in the presence or absence of the essential cofactor dihydronicotinamide riboside (NRH). In addition, fluorescence laser confocal microscopy was employed to determine whether selected analogues were taken up into cell nuclei.

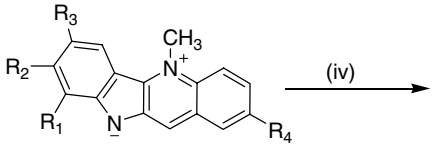
2. Results and discussion

2.1. Synthesis of nitrocryptolepine analogues

Halogenated analogues of **1** were prepared using a three-step methodology (Scheme 1) utilizing indoxyl-1,3-diacetate and isatin derivatives based on that of Holt and Petrow,[11] as previously described.[2,5] Nitrated halogenated derivatives of **1** were subsequently formed by direct nitration of the halogenated analogues by using either equivalent volumes of concentrated nitric acid (69%) and glacial acetic acid to form a mixture of two mono-nitro-regioisomers as shown in Scheme 2 or by using equivalent volumes of fuming nitric acid and glacial acetic acid to afford dinitro-analogues as shown in Scheme 3. The preparation of 7-nitro-, 9-nitro- and 7,9-dinitrocryptolepine analogues has been previously described by this research group[2,5] and it was found that when these positions are not blocked by other substituents, these positions are preferred on all occasions. The exact location of the nitro-groups on analogues of **1** was initially determined using 2D NMR experiments, in particular taking advantage of the through-space NOE enhancement of the protons of the *N*-CH_3 to the protons in position 4- and 6- as shown in Figure 1. For example, if the compound in question was a 7-nitro-regioisomer, then the *N*-CH_3 would show a through-space correlation to a doublet corresponding to H-4 and a singlet at H-6. However, if the compound in question was a 9-nitro isomer then the *N*-CH_3 would show a through-space correlation to a doublet corresponding to H-4 and also a doublet corresponding to H-6, since there is no substitution in position 7. Both ROESY and NOE difference NMR experiments on a high field 600 MHz spectrometer were successful in determining the positions of the nitro-groups which were further confirmed by the single crystal X-ray structures of two of the halogenated



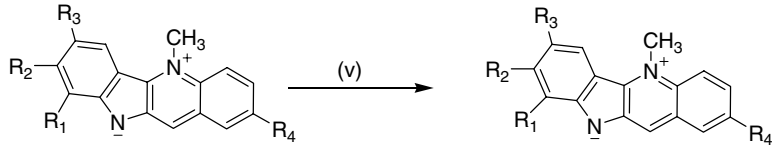
Scheme 1. Reagents and conditions: (i) KOH, N_2, 4 h; (ii) Ph_2O, reflux, 4 h; (iii) CH_3I, tetramethylene sulfone, 50 °C.



	R_1	R_2	R_3	R_4
1a	H	H	H	Cl
1b	H	H	H	F
1c	H	Cl	H	H
1d	H	H	Br	H

	R_1	R_2	R_3	R_4
2	H	H	NO_2	Cl
3	NO_2	H	H	Cl
5	H	H	NO_2	F
6	NO_2	H	H	F
8	H	Cl	NO_2	H
9	NO_2	Cl	H	H
10	H	NO_2	Br	H
11	NO_2	H	Br	H

Scheme 2. Reagents: (iv) HNO_3/HOAc (1:1), room temperature, overnight.



	R_1	R_2	R_3	R_4
1a	H	H	H	Cl
1b	H	H	H	F

	R_1	R_2	R_3	R_4
4	NO_2	H	NO_2	Cl
7	NO_2	H	NO_2	F

Scheme 3. Reagents: (v) $^{F}HNO_3$/HOAc (1:1), room temperature, overnight.

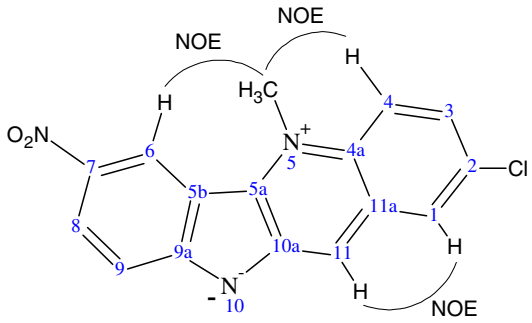


Figure 1. NOE connections from the *N*-CH_3 in **2**.

nitro-derivatives **2** and **5** (data not shown). Through-space correlations between H-1 and H-11 also provided starting points to work around the ring, which enabled all the protons in the spectra to be assigned (see Section 4). The carbon atoms in the ^{13}C spectra were assigned by using a heteronuclear two-dimensional HMQC experiment in order to determine the directly bonded $^{1}J_{C-H}$ correlations and HMBC spectra were acquired in order to identify $^{3}J_{C-H}$ correlations which were critical in assigning the quaternary carbons.

2.2. Biological evaluation of compounds

Table 1 shows the in-vitro cytotoxic activities of **1** and its derivatives against the three cell lines, RT112, H460 and BE, which were selected due to their content of the cytosolic reductase enzymes NQO1 and NQO2. Cryptolepine (**1**) as well as the analogues were all found to be cytotoxic with IC_{50} values ranging from 0.24 to 2.13 μM. Activation of nitro-analogues by the cytosolic reductase NQO2 was assessed by comparing the response of RT112 cells in the presence and absence of the required co-substrate NRH. If the nitro-derivatives of **1** were substrates for and activated by NQO2, then preferential toxicity in the presence of NRH should be obtained. CB 1954 was used as a positive control in this study and the response of RT112 cells clearly demonstrates that the activity of CB 1954 is enhanced in the presence of NRH. IC_{50} values for CB 1954 in the absence and presence of NRH were 88.75 and 0.15 μM, respectively, thus showing a 586-fold increase in cytotoxicity in the presence of NRH. In the case of **1** and its halogenated nitro-derivatives, no differences were found between the IC_{50} values obtained in the presence and absence of NRH, with the exception of 2-fluoro-7,9-dinitrocryptolepine (**7**). In this case, IC_{50} values were 0.45 and 0.19 μM in the absence and presence of NRH, respectively, indicating a 2.4-fold increase in cytotoxicity. The above compounds, with the exception of **3**, **4** and **5** were also assessed for their abilities to act as substrates for purified recombinant human NQO1 and the results obtained are shown in Table 2. As expected, the indoloquinone NQO1 substrate, EO9, was found to be readily metabolised by NQO1, while

Table 1. Cytotoxic activities of cryptolepine (**1**), nitrocryptolepine derivatives and standard drugs against R112 (high NQO2) cells in the absence and presence of NRH and against H460 (high NQO1) and BE (low NQO1) cells

Compound[a]	RT112[b]		H460[c]	BE[c]
	− Ve NRH	+ Ve NRH		
Cryptolepine (**1**)	0.82 ± 0.10	0.80 ± 0.15	1.45	1.28
2-Chloro-7-nitrocryptolepine (**2**)	0.41 ± 0.08	0.28 ± 0.01	0.46	0.95
2-Chloro-9-nitrocryptolepine (**3**)	0.88 ± 0.12	0.82 ± 0.01	—	—
2-Chloro-7,9-dinitrocryptolepine (**4**)	0.29 ± 0.06	0.23 ± 0.03	—	—
2-Fluoro-7-nitrocryptolepine (**5**)	2.13 ± 0.11	1.67 ± 0.06	—	—
2-Fluoro-9-nitrocryptolepine (**6**)	1.35 ± 0.21	1.10 ± 0.04	—	—
2-Fluoro-7,-9-dinitrocryptolepine (**7**)	0.45 ± 0.05	0.19 ± 0.01	—	—
8-Chloro-7-nitrocryptolepine (**8**)	1.03 ± 0.30	1.08 ± 0.43	—	—
8-Chloro-9-nitrocryptolepine (**9**)	0.76 ± 0.05	0.64 ± 0.01	1.88	1.48
7-Bromo-8-nitrocryptolepine (**10**)	0.36 ± 0.09	0.32 ± 0.04	0.38	0.58
7-Bromo-9-nitrocryptolepine (**11**)	0.24 ± 0.09	0.15 ± 0.04	0.59	0.78
CB 1954	88.75 ± 0.35	0.15 ± 0.01	—	—
EO9	—	—	0.0059	0.60

Results were expressed in terms of IC_{50} values (μM).
[a] Tested as hydrochloride salt with the exception of CB 1954 and EO9.
[b] $n = 3$.
[c] $n = 2$.

Table 2. Metabolism of cryptolepine (**1**), nitrocryptolepine derivatives and standard drugs by human recombinant NQO1

Compound[a]	Specific activity (μmol/min/mg)[b]
Cryptolepine (**1**)	4.53 ± 0.84
2-Chloro-7-nitrocryptolepine (**2**)	18.91 ± 0.46
2-Fluoro-9-nitrocryptolepine (**6**)	2.72 ± 0.17
2-Fluoro-7,9-dinitrocryptolepine (**7**)	2.96 ± 1.04
8-Chloro-9-nitrocryptolepine (**9**)	26.66 ± 2.48
7-Bromo-8-nitrocryptolepine (**10**)	13.91 ± 0.62
7-Bromo-9-nitrocryptolepine (**11**)	8.66 ± 1.34
CB 1954	3.56 ± 0.69
EO9	79.75 ± 1.34

[a] Cryptolepine and analogues tested as hydrochloride salts.
[b] $n = 3$.

cryptolepine and CB 1954 were not metabolised. The 8-chloro-9-nitro-analogue (**9**) was found to be the best substrate for NQO1 with a specific activity only 3-fold less than that of the well-established NQO1-activated compound EO9.[12] Analogues **2**, **10** and **11** were more slowly metabolised but **6** and **7** were found not to be substrates for NQO1. Derivatives of **1** that showed activity in the NQO1 substrate assay were then tested against the H460 and BE cell lines. These cell lines were chosen because of their differences in NQO1 content. The non-small cell lung carcinoma cell line H460 is known to contain high levels of NQO1, whereas the human colon carcinoma cell line BE is devoid of NQO1 activity due to the presence of a polymorphic variant that targets NQO1 protein for rapid degradation.[13] The results of the evaluation of **1** and those analogues that may act as substrates for NQO1 against H460 and BE cell lines are shown in Table 1. All of the compounds tested had similar activities against both cell lines, whereas EO9 was 100-fold more toxic to H460 cells than to BE cells as a result of bioactivation by NQO1. Although the ability of **9** to act as a substrate for NQO1 was 3-fold less than that of EO9, a modest enhancement of cytotoxicity might have been expected. It is possible that **9** is detoxified rather than activated by NQO1 in H460 cells[14] and it must also be noted that there are metabolic differences between the two cell lines other than their NQO1 activities/levels. In particular, 1- and 2-electron reduction by other enzymes (P450R and xanthine dehydrogenase/oxidase) is possible and should be considered when cytotoxicities in different cell lines are compared.[15] Another possibility is that **9** is activated by NQO1 but the activated molecule may be unable to reach its site of action.

The cellular incorporation and localisation of **1**, **2**, **7**, **10** and **11** into RT112 cells were examined using fluorescence laser confocal microscopy and the images obtained are shown in Figure 2. Cryptolepine (**1**) clearly shows nuclear localisation as expected on account of its DNA-intercalating properties,[7] but in contrast, the nitro-analogues were excluded from the nucleus (even after prolonged incubation of the cells), and were restricted to the cytoplasm. It is possible that the nitro- and/or halogen substituents prevent the transport of these analogues into the nucleus and/or prevent their intercalation into DNA. The results of previous work using thermodenaturation techniques with a number of nitro- and halogenated derivatives of **1** suggest that DNA intercalation may be blocked by substitution.[2]

3. Conclusion

All the analogues of **1** were shown to be relatively cytotoxic against the three cell lines assessed, with IC_{50} values <2 μM. Analysis of potential activation by NQO1 and NQO2 was assessed by comparing the response of the compounds with three cell lines which were selected due to their content of these cytosolic reductase enzymes. In the presence and absence of the required co-substrate NRH, the results of the biological evaluation of **1** and its derivatives suggest that these compounds

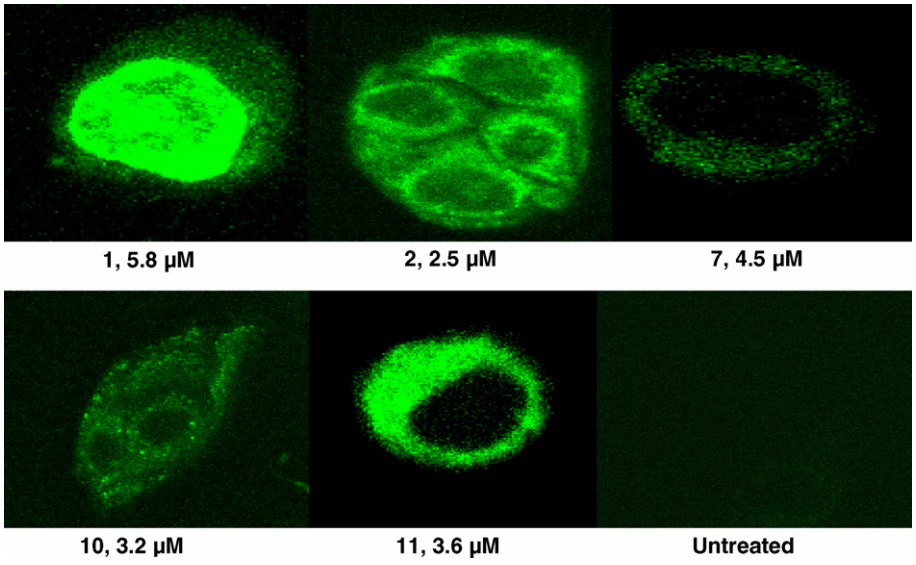


Figure 2. LCM images of selected cryptolepine derivatives and untreated cells using RT112 cells and a drug exposure time of 24 h. Images of **2** and **10** show several cells.

are not activated by NQO2 with the exception of **7**, where a modest (2.4-fold) potentiation of activity is seen in the presence of NRH in the RT112 cell line. Several compounds (**2**, **9**, **10** and **11**) were found to be substrates for NQO1 but for reasons that are unclear, they were not significantly more toxic to H460 (high NQO1 expressing) cells than to BE (low NQO1) cells although **2** was 2-fold more active against H460 than against BE cells.

Laser confocal microscopy demonstrated that selected halogenated nitro-derivatives (**2**, **7**, **10** and **11**) were restricted to the cytoplasm, in contrast to the parent compound **1**, which showed distinct nuclear localisation. This suggests that these analogues could be exerting their cytotoxic effect via a different mechanism to **1**, which has been shown to intercalate into DNA at GC rich sequences and inhibit topoisomerase II.[7] The results of this study demonstrate that nitrocryptolepine derivatives are cytotoxic and that some may be substrates for NQO1 or for NQO2. Only a limited series of compounds has been examined here and the synthesis and evaluation of more novel nitrocryptolepine analogues would be worthwhile. In addition, further studies to elucidate the cytotoxic mode(s) of action of these compounds are warranted.

4. Experimental

4.1. Chemistry

Reagents were purchased from Sigma–Aldrich (Gillingham, UK) and Lancaster (Morecambe, UK), unless otherwise stated. ^{1}H and ^{13}C NMR spectra were acquired on a JEOL ECA 600 spectrometer observing ^{1}H at 600.17 MHz and ^{13}C at 150.91 MHz. NOE experiments were carried out in DMSO-d_6 at 25 °C and chemical shifts were referenced to tetramethylsilane (TMS) as an internal standard (0 ppm). Mass spectrometry was carried out by Mr. Andrew Healey (University of Bradford) on a Micromass Quattro Ultima HPLC-MSMS electrospray spectrometer in either positive or negative ionisation modes, or alternatively using electron impact ionisation with a Finnigan MAT 90 spectrometer. C, H and N analyses were carried out by the Chemical and Materials Analysis Unit, University of Newcastle, UK, on a Carlo Erba 1106 elemental analyzer.

4.2. General method A: synthesis of mono-nitrated cryptolepine analogues

A halogenated cryptolepine hydrochloride analogue, glacial acetic acid and nitric acid (69%) were added together in a large vial and stirred at room temperature for 24 h. After the reaction time, distilled water (~5 ml) was carefully added and the mixture was then neutralised by the addition of saturated sodium hydroxide solution, whereupon the crude product precipitated as the free base. The precipitate was filtered under vacuum, washed with cold water and dried under vacuum at 45 °C overnight. TLC of the crude product showed the formation of two compounds, found to be the 7- and 9-nitrocryptolepine derivatives, unless otherwise stated. The isomers were separated and purified by column chromatography as the free base on a silica gel, unless otherwise stated, using a gradient elution of chloroform, concd ammonium hydroxide solution (1%) and increasing amounts of methanol (1–10%). The less-polar halogenated 7-nitro-compound eluted first as an orange coloured fraction, followed by the relatively more polar purple coloured 9-nitro isomer. The purified free bases were then converted to the hydrochloride salt by addition of HCl (10%) in methanol, followed by recrystallisation from chloroform/methanol/ethyl acetate.

4.2.1. 2-Chloro-7-nitrocryptolepine hydrochloride (2) and 2-chloro-9-nitrocryptolepine hydrochloride (3). Prepared according to general method A. 2-Chlorocryptolepine (**1a**, 100 mg, 0.33 mmol), glacial acetic acid (2 ml) and nitric acid (2 ml) were reacted together to form a mixture of the 7-nitro (**2**, 64 mg, 54.6%) and 9-nitro (**3**, 26 mg; 21.2%) cryptolepine isomers, which were separated and recrystallised to form their yellow hydrochloride salts.

4.2.1.1. Data for 2-chloro-7-nitrocryptolepine hydrochloride (2). Mp 261–262 °C with decomposition; ^{1}H NMR (DMSO-d_6) δ 14.02 (s, 1H, N-H), 9.59 (d, 1H, J = 2.15 Hz, H-6), 9.43 (s, 1H, H-11), 8.92 (d, 1H, J = 9.59 Hz, H-4), 8.83 (d, 1H, J = 2.43 Hz, H-1), 8.75 (dd, 1H, J = 7.02, 2.15 Hz, H-8), 8.27 (dd, 1H, J = 7.16, 2.43 Hz, H-3), 8.06 (d, 1H, J = 9.16 Hz, H-9), 5.14 (s, 3H, N^{+}-CH_3); ^{13}C NMR (DMSO-d_6) δ 148.72 (C-9a), 142.12 (O_2N-C-7), 139.42 (C-5a), 135.62 (C-4a), 135.14 (C-10a), 133.82 (C-3), 132.93 (Cl-C-2), 128.88 (C-1), 128.81 (C-8), 128.35 (C-11a), 126.39 (C-11), 123.99 (C-6), 121.16 (C-4), 114.64 (C-9), 113.88 (C-5b), 41.43 (CH_3); MS (ESI+) m/z (%) 312 $[M+H]^{+\cdot}$ (14), 266 (100), 265 (7), 254 (7), 231 (3); (EI) m/z (%) 311 $[M]^{+}$ (100), 281 (30), 265 (56), 253 (8), 229 (27); Acc Mass for $C_{16}H_{10}N_3O_2Cl$ calculated 311.0462; found: 311.0456. Anal. Calcd for $C_{16}H_{10}N_3O_2Cl{\cdot}1.15HCl$: C, 54.3; H, 3.18; N, 11.9. Found: C, 54.5; H, 3.34; N, 11.6.

4.2.1.2. Data for 2-chloro-9-nitrocryptolepine hydrochloride (3). Mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 13.55 (s, 1H, N-H), 9.38 (s, 1H, H-11), 9.34 (d, 1H, J = 8.31 Hz, H-6), 8.94 (s, 1H, H-1), 8.91 (d, 1H, J = 9.45 Hz, H-4), 8.87 (d, 1H, J = 8.02 Hz, H-8), 8.29 (d, 1H, J = 9.45 Hz, H-3) 7.74 (dd, 1H, J = 8.31, 8.02 Hz, H-7), 5.10 (s, 3H, N^{+}-Me); ^{13}C NMR (DMSO-d_6) δ 138.89 (C-9a), 138.62 (C-5a), 135.45 (C-4a), 135.15 (C-6), 133.87 (C-3), 133.74 (O_2N-C-9), 133.69 (C-10a), 132.97 (Cl-C-2), 130.37 (C-8), 129.19 (C-1), 128.35 (C-11a), 126.84 (C-11), 121.40 (C-7), 121.16 (C-4), 118.92 (C-5b), 41.54 (CH_3); MS (ESI+) m/z (%) 312 $[M+H]^{+\cdot}$ (100), 288 (11), 229 (4); (EI) m/z (%) 311 $[M]^{+}$ (55), 297 (100), 281 (30), 265 (28), 251 (41), 239 (11), 229 (16), 215 (23); Acc Mass for $C_{16}H_{10}N_3O_2Cl$ calculated 311.0462; found: 311.0470. Anal. Calcd for $C_{16}H_{10}N_3O_2Cl{\cdot}1.5HCl$: C, 52.5; H, 3.16; N, 11.5. Found: C, 52.6; H, 3.20; N, 11.1.

4.2.2. 2-Fluoro-7-nitro (5) and 2-fluoro-9-nitrocryptolepine hydrochloride (6). Prepared according to general method A. 2-Fluorocryptolepine (**1b**, 200 mg, 0.7 mmol), glacial acetic acid (5 ml) and nitric acid (5 ml) were reacted together to form a mixture of the 7-nitro (**4**, 140 mg, 60%)

and 9-nitro (**5**, 90 mg; 38.5%) isomers, which were separated and purified by column chromatography using Florisil® as the stationary phase and eluting with chloroform, ammonium hydroxide (1%) and increasing amounts of methanol (1–6%). The purified free bases were then converted to the hydrochloride salt by addition of HCl (10%) in methanol, followed by recrystallisation from chloroform/methanol/ethyl acetate.

4.2.2.1. Data for 2-fluoro-7-nitrocryptolepine hydrochloride (5). Mp >300 °C, decomposed 309 °C; ^{1}H NMR (DMSO-d_6) δ 14.47 (s, 1H, N-H), 9.59 (d, 1H, J = 2.00, H-6), 9.48 (s, 1-H, H-11), 8.99 (dd, 1H, J = 5.44, 4.58 Hz, H-4), 8.74 (dd, 1H, J = 9.16, 2.00 Hz, H-8), 8.54 (dd, 1H, J = 6.01, 2.86 Hz, H-1), 8.22 (t, 1H, J = 7.75 Hz, H-3), 8.07 (d, 1H, J = 9.16, H-9), 5.15 (s, 3H, N^+-CH_3); ^{13}C NMR (DMSO-d_6) δ 161.09–159.44 (C-2, $^1J_{C,F}$ = 244.2 Hz), 148.69 (C-9a), 141.97 (O_2N-C-7), 139.12 (C-5a), 135.65 (C-4a), 133.75 (C-10a), 128.88–128.80 (C-11a, $^3J_{C,F}$ = 11.6 Hz), 128.64 (C-8), 126.50–126.47 (C-11, $^4J_{C,F}$ = 5.8 Hz), 123.96 (C-6), 123.95–123.81 (C-3, $^2J_{C,F}$ = 26.0 Hz), 122.18–122.12 (C-4, $^3J_{C,F}$ = 10.1 Hz), 114.47 (C-4), 113.64–113.37 (C-1, $^2J_{C,F}$ = 23.1 Hz), 113.49 (C-5b), 41.12 (CH_3); MS (EI) *m/z* (%) 295 $[M]^+$ (100), 281 (20), 265 (33), 235 (12), 222 (10); Acc Mass for $C_{16}H_{10}N_3O_2F$ calculated 295.0757; found: 295.0759. Anal. Calcd for $C_{16}H_{10}N_3O_2F$·1.7HCl: C, 53.8; H, 3.28; N, 11.8. Found: C, 53.3; H, 3.70; N, 12.3.

4.2.2.2. Data for 2-fluoro-9-nitrocryptolepine hydrochloride (6). Mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 13.52 (s, 1H, N-H), 9.36 (s, 1H, H-11), 9.32 (d, 1H, J = 8.31, H-6), 8.97 (dd, 1H, J = 5.73, 4.30 Hz, H-4), 8.83 (d, 1H, J = 8.02 Hz, H-8), 8.60 (d, 1H, J = 8.88 Hz, H-1), 8.21 (dd, 1H, J = 8.02, 7.45 Hz, H-3), 7.69 (t, 1H, J = 8.02, 7.73, H-7), 5.09 (s, 3H, N^+-CH_3); ^{13}C NMR (DMSO-d_6) δ 161.09–159.43 (C-2, $^1J_{C,F}$ = 249.98 Hz), 140.06 (C-9a), 138.43 (C-5a), 138.38 (C-10a), 135.06 (C-6), 133.89 (C-4a), 133.78 (O_2N-C-9), 130.12 (C-8), 128.75–128.68 (C-11a, $^3J_{C,F}$ = 10.11 Hz), 126.98–126.94 (C-11, $^4J_{C,F}$ = 5.78 Hz), 123.72–123.55 (C-3, $^2J_{C,F}$ = 24.56 Hz), 122.12–122.07 (C-4, $^3J_{C,F}$ = 10.11 Hz), 120.98 (C-7), 119.02 (C-5b), 113.90–113.74 (C-1, $^2J_{C,F}$ = 24.56 Hz), 40.06 (CH_3); MS (EI) *m/z* (%) 295 $[M]^+$ (66), 281 (100), 265 (35), 235 (63), 222 (20), 208 (25), 162 (12); Acc Mass for $C_{16}H_{10}N_3O_2F$ calculated 295.0757; found: 295.0753. Anal. Calcd for $C_{16}H_{10}N_3O_2F$·1.85HCl: C, 53.0; H, 3.29; N, 11.6. Found: C, 53.0; H, 3.31; N, 11.8.

4.2.3. 8-Chloro-7-nitrocryptolepine hydrochloride (8) and 8-chloro-9-nitrocryptolepine hydrochloride (9). Prepared according to general method A. 8-Chlorocryptolepine (**1c**, 200 mg, 0.66 mmol), glacial acetic acid (5 ml) and nitric acid (5 ml) were reacted together to form a mixture of the 7-nitro (**8**, 95 mg, 40.9%) and 9-nitro (**9**, 33 mg; 13.6%) isomers, which were separated and purified by column chromatography using Florisil® as the stationary phase and eluting with chloroform, concd ammonium hydroxide solution (1%) and increasing amounts of methanol (1–6%). The purified free bases were then converted to the hydrochloride salt by addition of HCl (10%) in methanol, followed by recrystallisation from chloroform/methanol/ethyl acetate.

4.2.3.1. Data for 8-chloro-7-nitrocryptolepine hydrochloride (8). Mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 13.38 (s, 1H, N-H), 9.53 (d, 1H, J = 8.88 Hz, H-6), 9.48 (s, 1H, H-11) 8.84 (d, 1H, J = 9.16 Hz, H-4), 8.64 (d, 1H, J = 7.16 Hz, H-1), 8.25 (t, 1H, J = 8.30 Hz, H-3), 8.22 (s, 1H, H-9), 8.01 (t, 1H, J = 7.45 Hz, H-2), 5.06 (s, 3H, N^+-CH_3); ^{13}C NMR (DMSO-d_6) δ 146.74 (C-9a), 142.15 (O_2N-C-9), 138.48 (C-5a), 136.53 (C-4a), 135.46 (C-10a), 134.09 (C-3), 130.74 (C-1), 129.81 (Cl-C-8), 128.37 (C-2), 127.84 (C-11), 127.64 (C-11a), 125.09 (C-6), 118.64 (C-4), 115.99 (C-9), 112.68 (C-5b), 41.15 (CH_3); MS (EI) *m/z* (%) 313 [^{37}Cl sat of M^+] (33), 311 $[M]^+$ (100), 281 (35), 265 (55), 229 (36), 215 (15), 203 (10); Acc Mass for $C_{16}H_{10}N_3O_2Cl$ calculated 311.0461; found: 311.0458. Anal. Calcd for $C_{16}H_{10}N_3O_2Cl$·1.8HCl: C, 50.9; H, 3.15; N, 11.1. Found: C, 50.8; H, 3.39; N, 11.2.

4.2.3.2. Data for 8-chloro-9-nitrocryptolepine hydrochloride (9). Mp decomposed and melted 262 °C; ^{1}H NMR (DMSO-d_6) δ 13.62 (s, 1H, N-H), 9.40 (s, 1H, H-11), 9.16 (d, 1H, J = 8.88 Hz, H-6), 8.86 (d, 1H, J = 9.16 Hz, H-4), 8.69 (d, 1H, J = 8.31 Hz, H-1), 8.25 (t, 1H, J = 7.16 Hz, H-3), 8.03 (t, 1H, J = 7.45 Hz, H-2), 7.82 (d, 1H, J = 8.59, H-7), 5.07 (s, 3H, N^+-CH_3); ^{13}C NMR (DMSO-d_6) δ 139.07 (C-9a), 139.06 (Cl-C-8), 137.58 (C-5a), 136.94 (C-4a), 134.84 (C-10a), 134.35 (C-3), 133.34 (O_2N-C-9), 132.33 (C-6), 130.94 (C-1), 128.49 (C-2), 127.89 (C-11), 127.66 (C-11a), 123.76 (C-7), 118.71 (C-4), 117.36 (C-5b), 41.13 (CH_3); MS (EI) *m/z* (%) 313 [^{37}Cl sat of M^+] (33), 311 $[M]^+$ (100), 297 (95), 281 (52), 265 (45), 251 (49), 229 (46), 215 (35), 203 (15), 188 (15); Acc Mass for $C_{16}H_{10}N_3O_2Cl$ calculated 311.0462; found: 311.0467. Anal. Calcd for $C_{16}H_{10}N_3O_2Cl$·1.9HCl: C, 50.4; H, 3.12; N, 11.0. Found: C, 50.4; H, 3.48; N, 10.9.

4.2.4. 7-Bromo-8-nitrocryptolepine hydrochloride (10) and 7-bromo-9-nitrocryptolepine hydrochloride (11). Prepared according to general method A. 7-Bromocryptolepine (**1d**, 200 mg, 0.58 mmol), glacial acetic acid (5 ml) and nitric acid (5 ml) were reacted together to form a mixture of the 7-nitro (**10**, 70 mg, 30.7%) and 9-nitro (**11**, 27 mg; 12.1%) isomers, which were separated and recrystallised to form yellow hydrochloride salt.

4.2.4.1. 7-Bromo-8-nitrocryptolepine Hydrochloride (10). Mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 13.87 (s, 1H, N-H), 9.59 (d, 1H, J = 2.00 Hz, H-6), 9.45 (s, 1H, H-11), 8.99 (s, 1H, H-9), 8.88 (d, 1H, J = 9.16 Hz, H-4), 8.74 (d, 1H, J = 8.31 Hz, H-1), 8.29 (dd, 1H, J = 5.44, 1.43 Hz, H-3), 8.05 (t, 1H, J = 7.45 Hz, H-2), 5.14 (s, 3H, N^+-C H$_3$); ^{13}C NMR (DMSO-d_6) δ 146.89 (C-9a), 146.02 (C-10a), 142.27 (O_2N-C-8), 139.09 (C-5a), 137.04 (C-4a), 134.66 (C-3), 131.05 (C-1), 130.32 (C-9), 128.66 (C-2), 128.53 (C-11), 127.89 (C-11a), 122.89 (C-6), 118.77 (C-4), 114.98 (C-5b), 106.40 (Br-C-7), 41.07 (CH_3); MS (ESI+) *m/z* (%) 356 [^{79}M+H]$^{+\cdot}$ (5), 310 (100), 298 (5), 231 (20); Acc Mass for $C_{16}H_{11}BrN_3O_2$ calculated 356.0034; found: 356.0020. Anal. Calcd for $C_{16}H_{10}N_3O_2Br$·1.7HCl: C, 45.9; H, 2.8; N, 10.0. Found: 45.8; H, 3.14; N, 9.91.

4.2.4.2. Data for 7-bromo-9-nitrocryptolepine hydrochloride (11). Mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 13.64 (s, 1H, N-H), 9.50 (d, 1H, J = 1.72 Hz, H-6),

9.45 (s, 1H, H-11), 8.95 (d, 1H, J = 1.72 Hz, H-8), 8.90 (d, 1H, J = 9.16 Hz, H-4), 8.74 (dd, 1H, J = 7.02, 1.29 Hz, H-1), 8.29 (dd, 1H, J = 5.44, 1.43 Hz, H-3), 8.05 (t, 1H, J = 7.45 Hz, H-2), 5.11 (s, 3H, N^+-CH_3); ^{13}C NMR (DMSO-d_6) δ 137.15 (C-4a), 136.94 (C-9a), 136.50 (C-6), 134.89 (O_2N-C-9), 134.61 (C-3), 134.02 (C-10a), 131.88 (C-8), 131.09 (C-1), 128.69 (C-2), 128.44 (C-11), 127.89 (C-11a), 120.42 (C-5b), 118.91 (C-4), 112.60 (Br-C-7), 41.40 (CH_3); MS (ESI+) m/z (% RI) 356 [^{79}Br M+H]$^{+\cdot}$ (10), 326 (4), 310 (100), 298 (5), 231 (35), 219 (3); Acc Mass for $C_{16}H_{11}BrN_3O_2$ calculated 356.0034; found: 356.0038. Anal. Calcd for $C_{16}H_{10}N_3O_2Br$·HCl: C, 48.9; H, 2.81; N, 10.7. Found: C, 48.7; H, 2.80; N, 10.9.

4.3. General method B: di-nitration of halogenated cryptolepine derivatives

A halogenated cryptolepine hydrochloride derivative and glacial acetic acid were added together in a large vial and stirred in an ice bath for 10 min, whereupon an equivalent volume of fuming nitric acid was added dropwise. The mixture was allowed to stir in the ice bath for a further 10 min then at room temperature for a further 24 h. After the reaction time, distilled water (~5 ml) was carefully added and then the mixture was basified by the addition of a saturated sodium hydroxide solution, at which point the crude product precipitated as the free base. The precipitate was filtered under vacuum, washed with cold water and dried in the vacuum oven overnight. TLC of the crude product showed the formation of a halogenated dinitro-product as the major component. The crude product was purified by chromatography as the free base on a silica gel column, eluting with 5% MeOH:1% NH_4OH in $CHCl_3$ to afford the pure free base. This was subsequently converted to the yellow hydrochloride salt by addition of HCl (10%) in MeOH then recrystallised with $CHCl_3$/MeOH (3:1).

4.3.1. 2-Chloro-7,9-dinitrocryptolepine hydrochloride (4). Prepared according to general method B. 2-Chlorocryptolepine (**1a**, 100 mg, 0.33 mmol), glacial acetic acid (4 ml) and fuming nitric acid (4 ml) were reacted together to form yellow 2-chloro-7,9-dinitrocryptolepine hydrochloride (**3**, 39 mg, 30.3%); mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 14.12 (s, 1H, N-H), 9.89 (d, 1H, J = 2.00 Hz, H-6), 9.50 (s, 1H, H-11), 9.40 (d, 1H, J = 2.00 Hz, H-8), 8.97 (d, 1H, J = 2.29 Hz, H-1), 8.96 (d, 1H, J = 9.59 Hz, H-4), 8.33 (d, 1H, J = 9.59 Hz, H-3), 5.19 (s, 3H, N^+-CH_3); ^{13}C NMR (DMSO-d_6) δ 140.58 (C-9a), 140.42 (O_2N-C-7), 138.69 (C-5a), 136.10 (C-4a), 134.78 (C-3), 133.68 (Cl-C-2), 133.11 (O_2N-C-9), 129.56 (C-6), 129.49 (C-1), 129.00 (C-11), 128.56 (C-11a), 124.65 (C-8), 121.41 (C-4), 118.58 (C-5b), 41.75 (CH_3), not seen (C-10a); MS (ESI+) m/z (%) 312 [M+H]$^{+\cdot}$ (100), 288 (11), 229 (4); (EI) m/z (%) 311 [M]$^+$ (55), 297 (100), 281 (30), 265 (28), 251 (41), 239 (11), 229 (16), 215 (23); Acc Mass for $C_{16}H_{10}ClN_4O_4$ calculated 357.0391; found: 357.0400. Anal. Calcd for $C_{16}H_9N_4O_4Cl$·1.3HCl: C, 47.5; H, 2.55; N, 13.9. Found: C, 48.4; H, 2.80; N, 13.5.

4.3.2. 2-Fluoro-7,9-dinitrocryptolepine hydrochloride (7). Prepared according to general method B. 2-Fluorocryptolepine (**1b**, 200 mg, 0.7 mmol), glacial acetic acid (5 ml) and fuming nitric acid (5 ml) were reacted together to form yellow 2-fluoro-7,9-dinitrocryptolepine hydrochloride (**7**, 88 mg, 32.9%); mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 14.15 (s, 1H, N-H), 9.91 (d, 1H, J = 2.00 Hz, H-6), 9.52 (s, 1H, H-11), 9.40 (d, 1H, J = 2.0 Hz, H-8), 9.06 (dd, 1H, J = 5.58, 4.44 Hz, H-4), 8.69 (dd, 1H, J = 6.01, 2.86 Hz, H-1), 8.31 (ddd, 1H, J = 4.87, 3.01, 2.72 Hz, H-3), 5.21 (s, 3H, N^+-Me); ^{13}C NMR (DMSO-d_6) δ 161.52–159.86 (F-C-2, J = 249.98 Hz), 140.52 (C-9a), 140.25 (O_2N-C-7), 138.32 (C-5a), 134.78 (C-4a), 136.28 (C-10a), 133.00 (O_2N-C-9), 129.66–129.58 (C-11a, J = 11.56 Hz), 129.49 (C-6), 128.65–128.63 (C-11, J = 4.33 Hz), 125.09–124.91 (C-3, J = 27.45 Hz), 124.57 (C-8), 122.57–122.50 (C-4, J = 10.11 Hz), 118.66 (C-5b), 114.29–114.13 (C-1, J = 23.12 Hz), 41.99 (CH_3); MS (EI) m/z (%) 340 [M]$^+$ (48), 326 (100), 315 (8), 280 (20), 234 (58); Acc Mass for $C_{16}H_9N_4O_4F$ calculated 340.0609; found: 340.0608. Anal. Calcd for $C_{16}H_9N_4O_4F$·1.3HCl: C, 49.6; H, 2.68; N, 14.5. Found: C, 49.8; H, 2.88; N, 14.5.

4.4. Biological evaluation

4.4.1. Chemosensitivity testing using the colorimetric MTT (tetrazolium) assay.[16] Cells were routinely maintained as monolayer cultures in RPMI-1640 culture medium Supplemented with foetal calf serum (10%), sodium pyruvate (2 mM), L-glutamine (2 mM) and buffered with HEPES (25 mM). Chemosensitivity was assessed using the MTT assay. Briefly, cells at approximately 75% confluence were trypsinised and cells counted using a haemocytometer. One thousand cells were plated into each well of a 96-well-round bottomed culture plate (with the exception of lane 1 which contained only medium; this served as a blank for the spectrophotometer) with each well containing 150 μl of Supplemented medium. The cells were incubated overnight at 37 °C. The evaluation of compounds varied depending on whether or not compounds were being investigated as substrates for NQO1 or NQO2 as described below.

4.4.2. Drug exposure to RT112 cell lines (NQO2 studies). This assay is based upon the fact that NQO2 requires the co-substrate NRH in order to reduce substrates. If the nitro-derivatives of cryptolepines are substrates for and activated by NQO2, then preferential toxicity in the presence of NRH should be obtained. Following the overnight incubation described above, cells were treated with the drug in the presence and absence of the cofactor NRH (NRH was a gift from Professor Richard Knox, Morvus Technology, UK). The medium was completely removed from all 96 wells in the plate, and fresh medium was added to lane 1 (blank) and also in lane 2 (control). A control solution consisting of 0.1% DMSO in medium was added to lane 3, and lane 4 contained a NRH control solution (100 μM). All test compounds were dissolved in DMSO at 10 mM, aliquoted and routinely stored at −20 °C. A series of 2-fold serial dilutions of drugs were prepared in medium ranging from 10 μM down to 0.078 μM and these were added to lanes 5–12 in the presence or absence of NRH (100 μM). The number of wells used per drug concentration

was 8 (i.e., one lane of a 96-well plate) and each assay was repeated in triplicate. Results were expressed as the ratio of IC_{50} values obtained in the presence and absence of NRH and CB 1954 was used as a positive control compound.

4.4.3. Drug exposure to H460 and BE cell lines (NQO1 based studies). Cells were treated with selected compounds that had shown activity in the NQO1 substrate assay (described below). Following the overnight incubation period described above, medium was removed from all wells in the plate and lanes 1 and 2 were replenished with fresh medium, along with a 0.1% DMSO control in lane 3. 2-Fold dilutions of drugs were prepared in medium ranging from 10 μM down to 0.078 μM for the cryptolepine derivatives in both cell lines. However for EO9 (positive control compound), drug concentrations ranged from 50 μM down to 0.2 μM for the NQO1 deficient BE cell line, whereas concentrations ranged from 100 nM down to 0.4 nM for the high NQO1 H460 cell line. Each assay was repeated in duplicate and the results were expressed as the ratio of IC_{50} values obtained in BE cells to IC_{50} values in H460 cells.

4.4.4. NQO1 enzyme substrate assay. The ability of test compound to serve as substrates for NQO1 was determined according to previously published protocols.[12,17] Purified recombinant human NQO1 was provided by the Institute of Cancer Therapeutics (University of Bradford). NQO1 activity was assayed by measuring cytochrome *c* reduction at 550 nm on a CaryUV 50 (Varian, Australia) spectrophotometer. Each assay contained cytochrome *c* (70 μM), NADH (2 mM), purified NQO1 (1 μg) and drug concentrations of 10 μM in a final volume of 1 ml Tris–HCl (50 mM, pH 7.4) containing 0.14% BSA. Reactions were carried out at room temperature and started by the addition of NADH. A blank was initially acquired which consisted of buffer and cofactor (NADH), in the absence of cytochrome *c*. Background measurements (i.e., non-enzymatic reduction of cytochrome *c*) were taken in the absence of NQO1 for each drug concentration tested. These background values were subtracted from the values obtained in the presence of the NQO1 enzyme. Specific activities were calculated from the initial linear part of the reaction curve (30 s) and results were expressed in terms of the micromolar of cytochrome c reduced/min/mg of protein using a molar extinction coefficient of 21.1 $mM^{-1}\,cm^{-1}$.

4.4.5. Confocal laser scanning microscopy. RT112 cells were grown in borosilicate chambers at a concentration of 2×10^4 cells/ml and allowed to adhere overnight. They were then exposed to the test compound at approximately 10-fold IC_{50} concentration (μM), whereupon assessment of compound localisation into the cells was carried out using confocal laser scanning microscopy. This was performed using a Bio-Rad Microradiance Confocal Imaging System attached to a Nikon CM-800 microscope using LaserSharp 2000 software. Specimens were scanned using an argon laser with appropriate excitation and emission filters for the compounds (wavelength 488/500 nm) at 100% power, and an iris aperture of 6.3. Specimens were examined using a $60\times$ oil immersion objective lens. Optical sections of 512×512 pixels were captured at 50 lps and digital magnification of 2.4 using Kalman ($n = 4$) scanning settings. Simultaneous transmission images were also captured.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.06.062.

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