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Original article

Synthesis of 9-substituted derivatives of berberine as anti-HIV agents

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ABSTRACT

Naturally occurring protoberberine alkaloids, berberine and berberrubine along with 9-substituted derivatives of berberine were assessed for the anti-human immunodeficiency virus (HIV) activity. Berberine was found to be the most active compound with an EC_{50} of 0.13 μM against HIV-1 NL4.3 virus in CEM-GFP cell lines. Berberrubine and two other compounds were found to be less active than berberine, at the same time they were less toxic than berberine. Enzyme based assay suggested that the anti-HIV activity of berberine and its analogs might be due to RTase inhibitory activity and some additional mechanisms.

1. Introduction

Berberine (**1**) is a quaternary protoberberine alkaloid and is a major constituent of many medicinal plants of families Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, Annonaceae etc. [1]. Berberine possesses various biological activities like antibacterial [2,3], antifungal [4], antimalarial [5], antileishmanial [6], anticancer [7], antidiarrheal [8], cholesterol lowering effect [9] and hypoglycemic effect [10]. A number of 8, 9 and 13 substituted derivatives of berberine have been reported for different activities. Various analogs using different chain lengths and terminal amino groups have been synthesized to study the DNA-binding affinity or as G-quadruplex stabilizing ligands [11–17]. Similar types of berberine derivatives have been reported as acetylcholinesterase inhibitors [18].

Recently, Zha et al. have reported that HIV-protease inhibitors like amprenavir, atazanavir, lopinavir, and ritonavir have increased TNF-α and IL-6 expression in macrophages and thus caused stress. Berberine inhibits the expression of TNF-α and IL-6 in macrophages and thus effectively reduces stress in mouse macrophages induced by HIV-protease inhibitors. It was also concluded that berberine has the potential application as a complimentary therapeutic agent for HIV infection [19]. Berberine was found to be active against human cytomegalo virus (HCMV). The anti-HCMV activity of **1** (IC_{50} 0.68 μM) was better than ganciclovir (IC_{50} 0.91 μM), a standard drug for the treatment. The mechanism of action of **1** was proposed to be through intervention with intracellular events after virus penetration into the host cells and before viral DNA synthesis [20]. Earlier, anti-HIV reverse transcriptase (RT) activity of various protoberberine alkaloids (Fig. 1) including berberine was reported. Columbamine (**2**) iodide (IC_{50} 58 μg/ml), coptisine (**3**) picrate (IC_{50} 56 μg/ml), jatrorrhizine (**4**) chloride (IC_{50} 71 μg/ml) and berberine (**1**) chloride (IC_{50} 100 μg/ml) were found to be moderately active, whereas compounds that lacked quaternary nitrogen, e.g., tetrahydropalmatine and tetrahydroberberine, were inactive (25% inhibition at 200 μg/ml) against HIV RT. The unavailability of more diversified analogs of protoberberines prevented significant structure activity correlation in this series of alkaloids [21]. There have been no further attempts to study berberine derivatives as anti-HIV agents.

In continuation of our efforts to identify new anti-HIV lead molecules based on various natural product scaffolds [22,23], we synthesized various berberine-9-esters for evaluation of their anti-HIV activity.

2. Chemistry

Structural modifications at C-9 of berberine can be envisaged after converting the methoxy group to hydroxyl to obtain berberrubine. Various methods have been reported for the synthesis of berberrubine (**5**) from berberine. Hong et al. reported that stirring of **1** with urea at 200 °C yielded berberrubine after purification by

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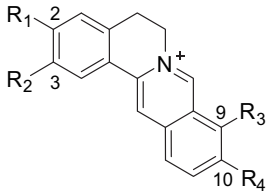


1: R_1+ R_2= OCH_2O, R_3= OCH_3, R_4= OCH_3
2: R_1= OH, R_2= OCH_3, R_3= OCH_3, R_4= OCH_3
3: R_1+ R_2= OCH_2O, R_3+ R_4= OCH_2O
4: R_1= OCH_3, R_2= OH, R_3= OCH_3, R_4= OCH_3

Fig. 1. Various protoberberine alkaloids.

column chromatography using $CHCl_3$: MeOH (4:1) [24]. Another, method reported by Das and Srinivas involves microwave irradiation [25]. The most efficient method was found to be vacuum pyrolysis, which does not require any specialized instrument or purification by column chromatography. It has been used widely for the synthesis of **5** [5,26]. In our study (Scheme 1), compound **1** was converted to **5** in 80–90% yield using vacuum pyrolysis. The next step involved acylation of **5** in a polar aprotic solvent using various acid chlorides. The desired final products were obtained in quantitative yield.

Different berberine-9-*O*-esters (**6**–**21**) were prepared using different acid chlorides as shown in Fig. 2. All synthesized compounds were identified by MS, IR, ^1H and ^{13}C NMR. All ester derivatives showed a strong C=O stretching absorption band in the region of 1730–1750 cm^{-1} in IR spectra and a peak in the range of δ 150–175 ppm in ^{13}C NMR spectrum. All other signals of berberine except $-OCH_3$ at C-9 were observed in all synthesized compounds.

3. Biology

All synthesized compounds were tested for their cytotoxicity in MTT assay before testing for cell based anti-HIV activity. Initially all the synthesized derivatives were tested for anti-HIV activity at highest non-toxic concentration. The results of cytotoxicity and anti-HIV activity are shown in Table 1. Three out of the 17 compounds showed more than 70% inhibition of HIV replication in CEM-GFP cells. These three compounds were analyzed further for determination of EC_{50} and CC_{50} in order to look for a potential lead molecule for further development. AZT was used as a positive control.

In order to find mechanism of action, three active compounds along with berberine were tested for HIV-1 RT inhibitory activity. The results of anti-HIV RT activity are shown in Table 2.

4. Results and discussion

The parent compound berberine (**1**) was found to be active against HIV-1 NL4.3 with an EC_{50} of 0.13 μM. Although berberrubine (**5**), a known natural product having free –OH group at C-9 was less active (EC_{50} of 2.8 μM) than **1**, interestingly it had better therapeutic index (TI) of >60, almost 3.75 times than berberine. So, attempts were made to introduce different substituents at C-9 of berberine by preparing various esters.

Benzoyl derivative (**6**) was weakly active compared to **5**. Substitution at 4′ in the benzoyl moiety by OMe (**9**) and SMe (**10**) gave differences in activity. Compound **9** was active (~ 44% inhibition) at 0.02 μM whereas **10** was inactive. In the case of substitution with five member hetero-aromatics with O and S as hetero-atom (12 and 13), the case was reverse. Compound 12 with furoyl moiety was weakly active at 18 μM concentration, while compound 13 with thiophenoyl moiety was more active with an EC50 value of 1.82 μM and TI of 50. 2-Napthoyl derivative (**7**) was moderately active whereas 1-napthoyl derivative (**8**) was inactive. Compound **21** with bulky substitution like 1-adamantoyl showed more than 60% inhibition at 1 μM concentration. This suggested that size and orientation of bulkiness at C-9 is important for anti-HIV activity.

Electron withdrawing groups like F and CF_3 on phenyl ring (**15**–**20**) at C-9 of berberrubine were introduced to see their effect on activity. 2,4-Difluoro substituted moiety (**15**) was the most active with an EC_{50} value of 0.95 μM and TI of 60.15. Change in the electronegativity at either position by CF_3 reduced anti-HIV activity (**17** and **18**). In addition, 3, 4 and 2, 6 di-substituted analogs were also inactive.

Berberine showed 94% inhibiton of HIV-1 RT at a concentration of 20 μg/reaction. All other screened compounds showed 99% inhibition of HIV-1 RT at the same concentration. The standard drug Nevirapine showed 95.5% inhibition at 0.1 μg/reaction concentration. The IC_{50} values showed that the most active compound (**1**) was about 50 times less active than the standard drug. Berberine and its derivatives showed low EC_{50} in cell based assay and high IC_{50} in RT assay indicating that they act by additional mechanisms, which remains to be clearly elucidated.

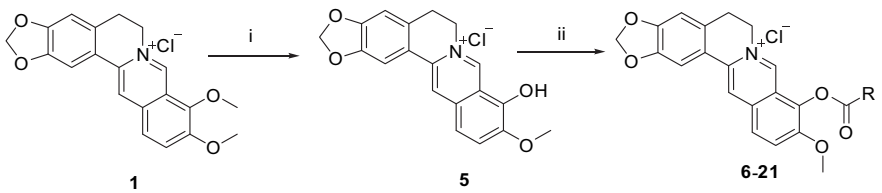
5. Conclusions

In summary, a new series of 9-substituted berberine derivatives has been synthesized. Naturally occurring berebrine (**1**) was found to be the most active against HIV-1 NL4.3 in CEM-GFP cell line. Berberrubine (**5**) a naturally occurring alkaloid was found to be less active and less toxic than berberine (**1**). Two other compounds (**13** and **15**) were also found to be less active and less toxic compared to berberine.

6. Experimental

6.1. General

^1H and ^{13}C NMR spectra were recorded on 400 MHz Bruker FT-NMR (Avance II 400) spectrometer using tetramethylsilane as an internal standard and the chemical shifts are reported in δ units. Mass spectra were recorded on either CIMS (LtQ, Thermo, USA) or MALDI MS (Bruker, USA). Merck silica gel 60 F_{254} plates were used for TLC. Developed plates were visualized by UV light. All chemicals were purchased from Sigma Aldrich. Solvents used for the chemical synthesis purchased from commercial sources were of analytical



Scheme 1. Reagents and conditions: i. 190 °C, 20–30 mm Hg, 2 h, ii. Acid chloride (RCOCl), CH_3CN, RT, 30 min.

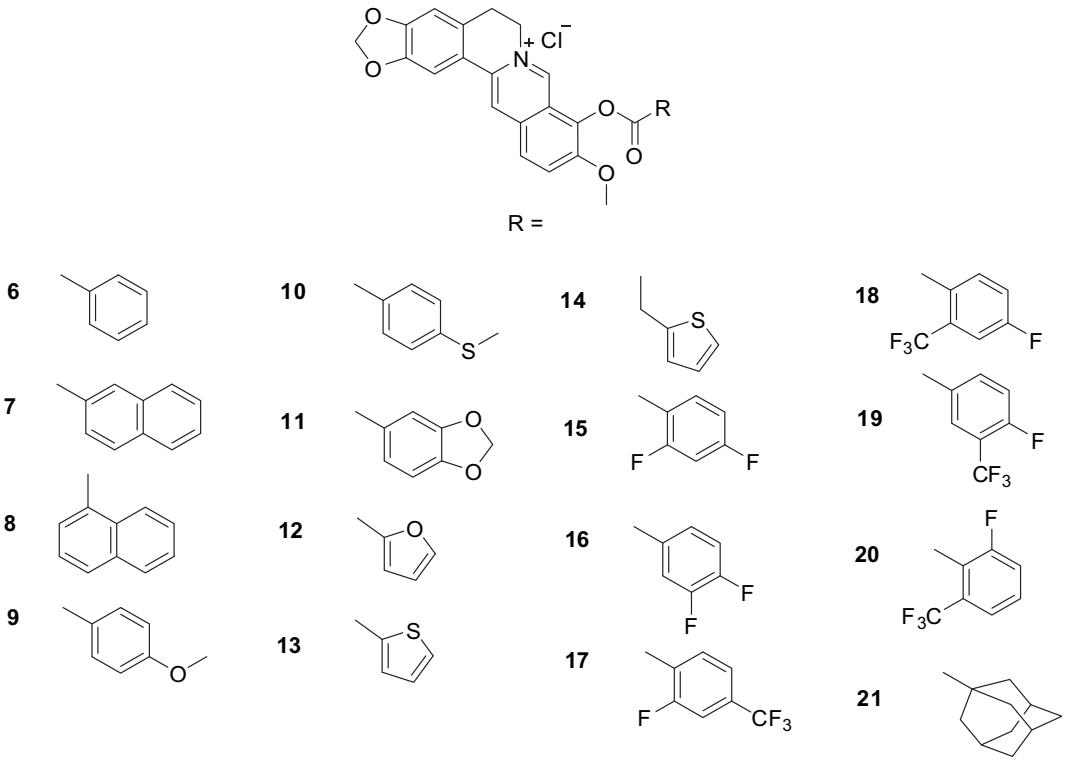


Fig. 2. Structures of synthesized 9-substituted berberine derivatives.

grade and were used without further purification. Concentration of solutions after reactions involved the use of a rotatory evaporator (Buchi, Switzerland) operating at a reduced pressure.

6.2. Chemistry

6.2.1. Procedure for synthesis of berberrubine (*5*)

Commercially available berberine (**1**, 1 g) chloride was heated at 190 °C in a vacuum oven under reduced pressure (20–30 mm Hg) for 1–2 h to obtain berberrubine (**5**, 885 mg) in 90% yield.

Table 1
Anti-HIV activity of 9-substituted berberine derivatives in CEM-GFP cell line.

Code	Conc. (μM)[a]	% Inhibition (p24)	EC_{50} (μM)[b]	CC_{50} (μM)[c]	TI[d]
1	0.29	66.0	0.13	2.09	16.07
5	15.52	76.95	2.8	169.2	60.42
6	4.69	37.2	ND	ND	ND
7	2.10	58.82	ND	ND	ND
8	1.05	0	ND	ND	ND
9	0.02	44.26	ND	ND	ND
10	5.30	0	ND	ND	ND
11	5.31	45.63	ND	ND	ND
12	18.03	22.62	ND	ND	ND
13	4.62	73.13	1.82	91.87	50.47
14	2.24	0	ND	ND	ND
15	4.32	71.65	0.95	57.14	60.15
16	21.65	0	ND	ND	ND
17	9.77	23.36	ND	ND	ND
18	4.88	0	ND	ND	ND
19	4.88	0	ND	ND	ND
20	1.95	0	ND	ND	ND
21	1.03	64.23	ND	ND	ND
AZT	5	89.75 ± 7.07	1.05 ± 0.07	24.06 ± 0.63	22.91

[a] Highest noncytotoxic concentration.
[b] EC_{50} = concentration of compound to achieve 50% inhibition of infected cells.
[c] CC_{50} = concentration of compound indicating 50% cytotoxicity in uninfected cells.
[d] TI = CC_{50}/EC_{50}; ND = Not determined.

*6.2.1.1. Berberine chloride (**1**).* Yellow solid; IR (KBr) ν_{max} 3405, 3049, 1598, 1567, 1506, 1480 cm^{-1}; ^1H NMR (CD_3OD) δ 9.76 (s, 1H), 8.66 (s, 1H), 8.09 (s, 1H), 7.99 (s, 1H), 7.62 (s, 1H), 6.95 (s, 1H), 6.09 (s, 2H), 4.93 (s, 2H), 4.20 (s, 3H), 4.09 (s, 3H), 3.30 (s, 1H), 3.25 (s, 1H); ^{13}C NMR (CD_3OD) δ 150.7, 150.6, 148.4, 145.0, 144.3, 138.2, 133.7, 130.4, 126.6, 123.1, 121.9, 120.4, 120.0, 108.0, 105.1, 102.2, 61.2, 56.2, 55.8, 26.8; MS: *m/z* 336 $[M - Cl]^+$.

*6.2.1.2. Beberrubine chloride (**5**).* Dark red solid; yield 80–90%; IR (KBr) ν_{max} 3365, 2901, 1634, 1614, 1568, 1543, 1505, 1471 cm^{-1}; ^1H NMR (CD_3OD) δ 9.19 (s, 1H), 7.90 (s, 1H), 7.45 (s, 1H), 7.34 (s, 1H), 6.80 (s, 2H), 6.00 (s, 2H), 4.85 (s, 2H), 3.83 (s, 3H), 3.09 (s, 2H); ^{13}C NMR (CD_3OD) δ 162.7, 149.6, 149.4, 148.0, 145.8, 134.0, 132.2, 129.2, 122.5, 121.4, 120.1, 118.2, 107.7, 106.9, 104.3, 101.8, 55.2, 54.1, 27.5, MS: *m/z* 323 $[M + 1\text{-}Cl]^+$ [5].

6.2.2. General procedure for synthesis of *6–21*

To the solution of **5** in CH_3CN various acid chlorides were added. Reaction mixture was allowed to stir for 30 min at room temperature. Solvent was evaporated under vacuum and crude product was washed with *n*-hexane/diethyl ether to yield **6–21** in 92–96% yield.

*6.2.2.1. 9-O-(Benzoyl) berberrubine chloride (**6**).* Yellowish brown solid; yield 94%; IR (KBr) ν_{max} 3406, 3014, 1745, 1609, 1507 cm^{-1}; ^1H NMR (CD_3OD) δ 9.72 (s, 1H), 8.84 (s, 1H), 8.32 (d, 2H, J = 6.8 Hz), 8.22 (d, 2H, J = 8.8 Hz), 7.78–7.64 (m, 4H), 6.95 (s, 1H), 6.11 (s, 2H), 4.91

Table 2
HIV-1 reverse transcriptase activity of 9-substituted berberine derivatives.

Compound	μg/reaction	% Inhibition	IC_{50} (μg/reaction)
1	20	94	2.1 ± 0.5
5	20	99	6.1 ± 0.1
13	20	99	3.8 ± 0.2
15	20	99	3.5 ± 0.1
Nevirapine	0.1	95.5	0.041

(s, 2H), 4.07 (s, 3H), 3.22 (s, 2H); ^{13}C NMR (CD_3OD) δ 163.9, 151.2, 151.0, 148.6, 143.9, 138.9, 134.4, 134.2, 133.8, 130.7, 130.3, 128.6, 128.1, 126.8, 125.6, 122.0, 120.7, 108.0, 105.3, 102.3, 56.4, 56.0, 26.6; MS: m/z 427 $[M + 1\text{-}Cl]^+$ [24].

*6.2.2.2. 9-O-(2-Napthoyl) berberrubine chloride (**7**).* Yellowish brown solid; yield 96%; IR (KBr) ν_{max} 3826, 3739, 3616, 1735, 1613, 1508 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.75 (s, 1H), 8.96 (s, 1H), 8.79 (s, 1H), 8.26–8.18 (m, 3H), 8.06 (t, 2H, J = 8.6 Hz), 8.00 (d, 2H, J = 8.4 Hz), 7.70 (t, 1H, J = 7.8 Hz), 7.66 (s, 1H), 7.62 (t, 1H, J = 7.4 Hz), 6.92 (s, 1H), 6.09 (s, 2H), 4.89 (t, J = 6.0 Hz), 4.05 (s, 3H), 3.20 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 165.5, 152.6, 152.4, 150.0, 145.3, 140.2, 137.6, 135.8, 135.1, 133.9, 133.8, 132.0, 130.6, 130.4, 129.8, 128.3, 128.3, 127.0, 126.6, 126.5, 123.3, 122.0, 121.6, 109.4, 106.7, 103.7, 57.9, 57.3, 28.0; MS: m/z 477 $[M + 1\text{-}Cl]^+$.

*6.2.2.3. 9-O-(1-Napthoyl) berberrubine chloride (**8**).* Yellowish brown solid; yield 92%; IR (KBr) ν_{max} 3415, 3020, 1734, 1609, 1507 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.79 (s, 1H), 9.00 (d, 1H, J = 9.5 Hz), 8.86 (s, 1H), 8.75 (d, 1H, J = 7.2 Hz), 8.32–8.27 (overlapped signal, 2H), 8.03 (d, 1H, J = 8.4 Hz), 7.72–7.52 (m, 4H), 6.95 (s, 1H), 6.12 (s, 2H), 4.90 (t, J = 6.0 Hz), 4.07 (s, 3H), 3.22 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 168.1, 155.2, 154.9, 152.5, 148.0, 143.0, 138.9, 138.5, 138.0, 137.8, 136.0, 135.6, 134.6, 132.6, 132.0, 130.7, 130.3, 129.6, 129.0, 128.4, 128.0, 126.0, 124.6, 124.2, 111.9, 109.2, 106.2, 60.4, 59.9, 30.6; MS: m/z 477 $[M + 1\text{-}Cl]^+$.

*6.2.2.4. 9-O-(4-Methoxybenzoyl) berberrubine chloride (**9**).* Yellowish brown solid; yield 96%; IR (KBr) ν_{max} 3343, 1734, 1605, 1508 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.68 (s, 1H), 8.81 (s, 1H), 8.26–8.18 (m, 4H), 7.68 (s, 1H), 7.14 (d, 2H, J = 8.4 Hz), 6.90 (s, 1H), 6.10 (s, 2H), 4.90 (t, J = 6.0 Hz), 4.05 (s, 3H), 3.94 (s, 3H), 3.21 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 164.9, 163.7, 151.2, 150.9, 148.6, 143.9, 138.8, 134.5, 133.8, 132.5, 131.4, 130.6, 126.6, 125.6, 122.0, 120.6, 120.3, 120.0, 113.9, 113.2, 108.0, 105.2, 102.3, 56.4, 55.9, 54.9, 26.6; MS: m/z 457 $[M + 1\text{-}Cl]^+$.

*6.2.2.5. 9-O-(4-Methylthiobenzoyl) berberrubine chloride (**10**).* Yellowish brown solid; yield 92%; IR (KBr) ν_{max} 3416, 1733, 1608, 1593, 1506 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.72 (s, 1H), 8.84 (s, 1H), 8.29–8.22 (m, 4H), 7.70 (s, 1H), 7.47 (d, 2H, J = 6.8 Hz), 6.96 (s, 1H), 6.12 (s, 2H), 4.90 (brs), 4.07 (s, 3H), 3.23 (t, 2H, J = 6.4 Hz), 2.61 (s, 3H); ^{13}C NMR (CD_3OD) δ 163.7, 151.2, 151.0, 148.6, 148.3, 143.9, 138.8, 134.4, 133.7, 130.6, 130.5, 126.7, 125.5, 124.7, 124.5, 123.7, 122.0, 120.6, 120.3, 108.0, 105.2, 102.3, 56.3, 55.9, 26.6, 13.1; MS: m/z 473 $[M + 1\text{-}Cl]^+$.

*6.2.2.6. 9-O-(Piperonyl) berberrubine chloride (**11**).* Yellow solid; yield 95%; IR (KBr) ν_{max} 3434, 2925, 1734, 1610, 1507 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.70 (s, 1H), 8.84 (s, 1H), 8.27 (d, 1H, J = 9.2 Hz), 8.23 (d, 1H, J = 9.2 Hz), 7.94 (d, 1H, J = 8.0 Hz), 7.70 (s, 2H), 7.06 (d, 1H, J = 8.4 Hz), 6.96 (s, 1H), 6.15 (s, 2H), 6.12 (s, 2H), 4.89 (t, J = 6.4 Hz), 4.07 (s, 3H), 3.22 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 164.7, 154.6, 152.7, 152.4, 150.0, 149.7, 145.3, 140.3, 135.9, 135.2, 132.1, 128.1, 128.1, 127.0, 123.4, 123.1, 122.0, 121.7, 111.0, 109.4, 109.4, 106.7, 103.9, 103.7, 57.8, 57.3, 28.0; MS: m/z 471 $[M + 1\text{-}Cl]^+$.

*6.2.2.7. 9-O-(2-Furoyl) berberrubine chloride (**12**).* Yellow solid; yield 93%; IR (KBr) ν_{max} 3414, 3022, 1750, 1621, 1609, 1506 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.73 (s, 1H), 8.83 (s, 1H), 8.27 (d, 1H, J = 8.8 Hz), 8.22 (d, 1H, J = 9.2 Hz), 7.97 (s, 1H), 7.69 (s, 1H), 7.66 (s, 1H, J = 3.2 Hz), 6.96 (s, 1H), 6.80 (s, 1H), 6.11 (s, 2H), 4.91 (t, J = 5.6 Hz), 4.08 (s, 3H), 3.23 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 151.1, 151.0, 148.6, 148.6, 143.8, 142.8, 138.5, 133.7, 133.4, 130.7, 127.0, 125.6, 121.9, 120.9, 120.6, 120.3, 112.4, 108.0, 105.3, 102.3, 56.4, 56.0, 26.6; MS: m/z 417 $[M + 1\text{-}Cl]^+$.

*6.2.2.8. 9-O-(2-Thiopheneoyl) berberrubine chloride (**13**).* Yellowish brown solid; yield 95%; IR (KBr) ν_{max} 3403, 2925, 1734, 1609, 1507, 1480 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.72 (s, 1H), 8.84 (s, 1H), 8.27 (d, 1H, J = 9.2 Hz), 8.22 (d, 1H, J = 9.2 Hz), 8.16 (d, 1H, J = 3.6 Hz), 8.01 (d, 1H, J = 4.8 Hz), 7.69 (s, 1H), 7.34 (t, 1H, J = 4.4 Hz), 6.95 (s, 1H), 6.11 (s, 2H), 4.91 (t, J = 6.4 Hz), 4.07 (s, 3H), 3.22 (t, 2H, J = 6.4 Hz); ^{13}C NMR (CD_3OD) δ 159.2, 151.3, 151.0, 148.6, 143.8, 139.0, 135.8, 135.1, 133.8, 133.7, 130.9, 130.7, 128.3, 127.0, 125.6, 121.9120.7, 120.3, 108.0, 105.2, 102.3, 56.4, 56.0, 26.6; MS: m/z 433 $[M + 1\text{-}Cl]^+$.

*6.2.2.9. 9-O-(2-Thiopheneacetonyl) berberrubine chloride (**14**).* Brown solid; yield 94%; IR (KBr) ν_{max} 3415, 1782, 1607, 1508 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.69 (s, 1H), 8.78 (s, 1H), 8.21 (d, 1H, J = 9.2 Hz), 8.15 (d, 1H, J = 9.2 Hz), 7.66 (s, 1H), 7.40 (d, 1H, J = 5.2 Hz), 7.18 (d, 1H, J = 6.3 Hz), 7.07 (t, 1H, J = 3.4 Hz), 6.97 (s, 1H), 6.11 (s, 2H), 4.89 (brs), 4.45 (s, 2H), 4.02 (s, 3H), 3.23 (t, 2H, J = 6.4 Hz); ^{13}C NMR (CD_3OD) δ 168.1, 151.0, 151.0, 148.5, 143.7, 138.5, 134.1, 133.6, 130.6, 127.4, 126.8, 126.6, 125.5, 125.2, 121.6, 120.5, 120.2, 108.0, 105.2, 102.3, 56.3, 56.0, 33.9, 26.6; MS: m/z 447 $[M + 1\text{-}Cl]^+$.

*6.2.2.10. 9-O-(2,4-Diflourobenzoyl) berberrubine chloride (**15**).* Yellow solid; yield 93%; IR (KBr) ν_{max} 3368, 3026, 1751, 1611, 1568, 1506 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.78 (s, 1H), 8.85 (s, 1H), 8.37 (dd, 1H, J = 8.4, 14.8 Hz), 8.29 (d, 1H, J = 9.2 Hz), 8.24 (d, 1H, J = 9.2 Hz), 7.70 (s, 1H), 7.31–7.23 (m, 2H), 6.94 (s, 1H), 6.11 (s, 2H), 4.92 (t, J = 6.0 Hz), 4.09 (s, 3H), 3.23 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 162.0, 152.5, 152.4, 150.0, 145.3, 140.4, 136.3, 136.2, 135.2, 135.1, 132.1, 128.4, 127.0, 123.2, 122.1, 121.7, 113.4, 113.2, 109.4, 107.0, 106.7, 106.4, 103.8, 57.9, 57.3, 28.0; MS: m/z 463 $[M + 1\text{-}Cl]^+$.

*6.2.2.11. 9-O-(3,4-Diflourobenzoyl) berberrubine chloride (**16**).* Yellowish brown solid; yield 94%; IR (KBr) ν_{max} 3410, 3022, 2924, 1752, 1611, 1507 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.78 (s, 1H), 8.85 (s, 1H), 8.31–8.18 (m, 4H), 7.70 (s, 1H), 7.57 (m, 1H), 6.96 (s, 1H), 6.12 (s, 2H), 4.92 (t, J = 6.4 Hz), 4.07 (s, 3H), 3.23 (t, 2H, J = 6.4 Hz); ^{13}C NMR (CD_3OD) δ 165.9, 155.0, 155.0, 152.5, 147.8, 143.0, 137.9, 137.7, 134.6, 131.9, 131.0, 129.5, 125.7, 124.6, 124.2, 123.5, 123.3, 121.9, 121.7, 111.9, 109.2, 106.3, 60.4, 59.8, 30.6; MS: m/z 463 $[M + 1\text{-}Cl]^+$.

*6.2.2.12. 9-O-(2-Flouro-4-triflouromethylbenzoyl) berberrubine chloride (**17**).* Brownish yellow solid; yield 96%; IR (KBr) ν_{max} 3425, 2925, 1756, 1609, 1507 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.83 (s, 1H), 8.85 (s, 1H), 8.49 (t, 1H, J = 7.2 Hz), 8.33 (d, 1H, J = 12.8 Hz), 8.27 (d, 1H, J = 12.0 Hz), 7.79 (d, 2H, J = 10.0 Hz), 7.69 (s, 1H), 6.96 (s, 1H), 6.11 (s, 2H), 4.86 (brs), 4.08 (s, 3H), 3.17 (brs, 2H); ^{13}C NMR (CD_3OD) δ 165.7, 162.2, 161.7, 152.8, 150.4, 145.7, 140.9, 138.9, 135.7, 135.6, 135.3, 132.5, 129.1, 127.4, 126.4, 123.5, 123.0, 122.5, 122.0, 116.6, 116.3, 109.8, 107.1, 104.2, 58.3, 57.8, 28.5; MS: 513 $[M + 1\text{-}Cl]^+$.

*6.2.2.13. 9-O-(4-Flouro-2-triflouromethylbenzoyl) berberrubine chloride (**18**).* Yellowish brown solid; yield 95%; IR (KBr) ν_{max} 3423, 3027, 1768, 1611, 1506 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.72 (s, 1H), 8.86 (s, 1H), 8.62 (t, 1H, J = 6.8 Hz), 8.31 (d, 1H, J = 9.2 Hz), 8.26 (d, 1H, J = 9.2 Hz), 7.80 (d, 1H, J = 9.2 Hz), 7.70 (s, 2H), 6.97 (s, 1H), 6.12 (s, 2H), 4.94 (t, J = 6.4 Hz), 4.08 (s, 3H), 3.25 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 168.0, 164.6, 162.7, 152.5, 150.1, 144.9, 140.6, 137.0, 136.8, 135.3, 135.0, 133.9, 132.2, 128.7, 127.1, 125.7, 123.1, 122.2, 121.7, 120.6, 120.4, 117.0, 116.9, 116.6, 116.5, 109.5, 106.8, 103.8, 57.9, 57.5, 28.1; MS: m/z 513 $[M + 1\text{-}Cl]^+$.

*6.2.2.14. 9-O-(4-Flouro-3-triflouromethylbenzoyl) berberrubine chloride (**19**).* Yellowish brown solid; yield 93%; IR (KBr) ν_{max} 3405, 3026, 1757, 1709, 1623, 1609, 1506 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.82 (s, 1H), 8.86 (s, 1H), 8.61 (m, 2H), 8.32–8.27 (m, 2H), 7.70 (s,1H), 7.67 (t, 1H, J = 10.0 Hz), 6.96 (s, 1H), 6.11 (s, 2H), 4.91 (t, J = 6.0 Hz), 4.08

(s, 3H), 3.23 (t, 2H, J = 5.6 Hz); ^{13}C NMR (CD_3OD) δ 166.7, 163.6, 163.2, 152.9, 150.4, 145.8, 140.8, 139.0, 138.9, 137.8, 137.7, 135.5, 132.4, 131.5, 129.0, 127.3, 127.0, 123.5122.4, 122.1, 119.9, 119.6, 118.7, 109.8, 107.0, 104.1, 58.3, 57.7 28.4; MS: m/z 513 $[M + 1\text{-}Cl]^+$.

*6.2.2.15. 9-O-(2-Flouro-6-triflouromethylbenzoyl) berberrubine chloride (**20**).* Yellowish brown solid; yield 95%; IR (KBr) ν_{max} 3406, 3026, 1768, 1612, 1507 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.72 (s, 1H), 8.86 (s, 1H), 8.62 (t, 1H, J = 7.6 Hz), 8.31 (d, 1H, J = 9.2 Hz), 8.26 (d, 1H, J = 9.2 Hz), 7.81 (d, 1H, J = 8.8 Hz), 7.70 (s, 2H), 6.97 (s, 1H), 6.11 (s, 2H), 4.94 (t, J = 6.0 Hz), 4.07 (s, 3H), 3.25 (t, 2H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 152.9, 145.3, 137.2, 129.1, 127.4, 122.6, 109.8, 107.1, 104.1, 58.2, 57.8, 28.4; MS: m/z 513 $[M + 1\text{-}Cl]^+$.

*6.2.2.16. 9-O-(1-Adamantoyl) berberrubine chloride (**21**).* Yellowish brown solid; yield 96%; IR (KBr) ν_{max} 3414, 2906, 2850, 1750, 1609, 1507 cm^{-1}; ^{1}H NMR (CD_3OD) δ 9.38 (s, 1H), 8.80 (s, 1H), 8.20 (d, 1H, J = 9.2 Hz), 8.15 (d, 1H, J = 9.2 Hz), 7.67 (s, 1H), 6.96 (s, 1H), 6.11 (s, 2H), 4.94 (t, J = 6.4 Hz), 4.05 (s, 3H), 3.22 (t, 2H, J = 6.0 Hz), 2.25–1.88 (m, 15H, adamantyl protons); ^{13}C NMR (CD_3OD) δ 174.7, 151.0, 150.8, 148.5, 143.4, 138.7, 134.7, 133.7, 130.7, 126.4, 125.5, 121.7, 120.7, 120.3, 108.0, 105.2, 102.3, 56.2, 56.2, 41.5, 38.7, 38.6, 36.1, 35.9, 28.0, 26.7; MS: m/z 485 $[M + 1\text{-}Cl]^+$.

6.3. Biology

6.3.1. Cell cytotoxicity assay using MTT

Cytotoxicity of potential candidates was assayed using MTT based Cell proliferation assay kit (Roche) in the CEM-GFP cell line according to the manufacturer's protocol. Briefly, 2×10^4 cells/well were seeded in 96-well plate; samples were then added into the wells at different concentrations keeping untreated and vehicle treated wells as controls. After 48 h incubation, 10 μL of MTT reagent (5 mg/ml) was added in to the wells to allow the reaction. Formazan crystals produced during the reaction were solubilized and color development was read at 540 nm.

6.3.2. Anti-HIV screening in CEM-GFP cells

Human CD4+ T cell line, CEM-GFP cells were infected with HIV-1 NL4.3 virus at a multiplicity of infection (MOI) of 0.05 using the standard protocol previously published from the lab [27,28]. The infection was monitored by GFP visualization under the microscope. The cells were incubated with samples for up to 7 days post infection. Virus production was assayed in the culture supernatant on day-7 post infection by p24 antigen capture ELISA (Perkin Elmer Life Science, USA). AZT was used as a positive control.

6.3.3. HIV-1 reverse transcriptase inhibition assay

A colorimetric assay was performed to analyze the HIV-1 RT inhibitory activity of the synthesized compounds using HIV-1 RT assay kit (Roche, Germany) according to manufacturer's protocol. Nevirapine was used as a positive control.

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