AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Amine-Linked Flavonoids as Agents against Cutaneous Leishmaniasis

Chin-Fung Chan,[a] Zhen Liu,[a] Iris L. K. Wong,[a] Xianliang Zhao,[a] Zaofeng Yang,[c] Jiale Zheng,[c] Marianne M. Lee,[c] Michael K. Chan,[c] Tak Hang Chan,[a,b] Larry M. C. Chow[a]

[a]Department of Applied Biology and Chemical Technology and State Key Laboratory of Chemical Biology and Drug Discovery, The Hong Kong Polytechnic University, Hong Kong SAR, China

[b]Department of Chemistry, McGill University, Montreal, Quebec, Canada

[c]School of Life Sciences and Center of Novel Biomaterials, The Chinese University of Hong Kong, Hong Kong SAR, China

Chin-Fung Chan and Zhen Liu contributed equally to this work. Author order was determined alphabetically.

ABSTRACT We have designed, synthesized, and characterized a library of 38 novel flavonoid compounds linked with amines. Some of these amine-linked flavonoids have potent *in vitro* activity against parasites that cause cutaneous leishmaniasis, a tropical disease endemic in 80 countries worldwide. The most promising candidate, FM09h, was highly active, with a 50% inhibitory concentration (IC_{50}) of 0.3 μM against *Leishmania amazonensis*, *L. tropica*, and *L. braziliensis* amastigotes. It was metabolically stable, with 39% and 66% of FM09h remaining after 30-minute incubation with human and rat liver microsomes, respectively. In *L. amazonensis* LV78 cutaneous leishmaniasis mouse model, intralesional injection of FM09h (10 mg/kg, once every 4 days for 8 times) demonstrated promising effect in reducing the footpad lesion thickness by 72%, displaying an efficacy comparable to that of sodium stibogluconate (SSG) (63%).

KEYWORDS *Leishmania*, promastigote, amastigote, cutaneous leishmaniasis, flavonoids, bioflavonoids, antileishmanial

Leishmaniasis is a vector-borne parasitic disease caused by protozoan parasites *Leishmania*, which are transmitted by the bite of infected sandflies. There are 3 main forms of the disease: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). VL is fatal if left untreated. It is characterized by fever, weight loss, enlargement of the spleen and liver, and anemia. An estimated 50,000 to 90,000 new cases of VL occur worldwide annually. CL is the most common form of leishmaniasis with an estimated 600,000 to 1 million new cases worldwide annually (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis). CL causes skin lesions, mainly ulcers, on exposed parts of the body, leaving lifelong scars and serious disability. MCL, which leads to destruction of mucous membranes of the nose, mouth, and throat, appears to be much less prevalent. Leishmaniasis is found in 88 countries, mainly in South and Central America, Africa, Asia, and southern Europe. Leishmaniasis ranks as a leading neglected tropical disease (NTD) in terms of mortality and morbidity that caused 50,000 deaths and over 2.3 million disability adjusted life years (1). It is estimated that a total of 399 and 556 million people living in areas of endemicity are at risk of cutaneous and visceral leishmaniasis, respectively (1).

Without available vaccines, the treatment of leishmaniasis mainly relies on chemotherapy. Many of the current medications, however, suffer from various limitations (2). Resistance to sodium stibogluconate (SSG), which has been used for over 5 decades, has been documented (3). For pentamidine, in addition to its possible side effect of hypoglycemia and hypotension, drug resistance has emerged (2). Amphotericin B has known infusion-related side effects associated with renal diseases and its lipid-

Citation Chan C-F, Liu Z, Wong ILK, Zhao X, Yang Z, Zheng J, Lee MM, Chan MK, Chan TH, Chow LMC. 2021. Amine-linked flavonoids as agents against cutaneous leishmaniasis. Antimicrob Agents Chemother 65:e02165-20. https://doi.org/10.1128/AAC.02165-20.

Address correspondence to Tak Hang Chan, tak-hang.chan@polyu.edu.hk, or Larry M. C. Chow, larry.chow@polyu.edu.hk.

Received 15 October 2020
Returned for modification 27 December 2020
Accepted 19 February 2021
Accepted manuscript posted online 8 March 2021
Published 19 April 2021

associated formulations are relatively expensive (4). The major side effects of miltefosine were gastrointestinal, vomiting and diarrhea (2). In addition, miltefosine refractory cases have been reported (5). Injectable paromomycin has been used for visceral leishmaniasis (6). It has potential renal and cranial nerve toxicity (7). Without new drugs, treatment of leishmaniasis can only rely on combination therapy to increase the treatment efficacy, reduce toxicity, and limit the emergence of drug resistance (8). In addition, many of the current medications require parenteral injection as well as hospitalization. Only miltefosine and paromomycin can be used in oral and topical administration, respectively. Therefore, there is a need to develop new candidate drugs for the treatment of leishmaniasis.

Flavonoids are natural and abundant polyphenolic compounds commonly found in fruits, vegetables, nuts, stems, flowers, wine, and tea (9). They are common components of normal human food and are generally considered to be safe for consumption. Their consumption has been proposed to be associated with a wide range of beneficial properties for human health, such as improving cognitive capabilities and related neurodegenerative disorders, including Alzheimer's disease (10). They have also been shown to have anti-inflammatory (11), anticancer (12), antibacterial (13), antifungal, and antiviral activities (13). The flavonoids apigenin and quercetin have been found to kill *Leishmania* promastigotes and amastigotes (14, 15). We have recently reported that some synthetic flavonoid dimers can reverse pentamidine and SSG resistance in *Leishmania* (16), and they can also combine with quinacrine to reverse pentamidine resistance in a synergistic manner (17). In particular, we discovered that some amine-linked flavonoid dimers have potent antileishmanial activities *in vitro*, with FD39 (chemical structure shown in Fig. 1A) having a 50% inhibitory concentration (IC_{50}) of 0.63 μM against intracellular amastigotes (18), and *in vivo* for CL (19). The high molecular weight and metabolic instability of FD39, however, hampered its further development.

Here, we developed a new series of amine-containing flavonoid monomers to address the high molecular weight issue as well as the metabolic stability problem encountered in FD39. A collection of 38 amine-containing flavonoids was designed, synthesized, and characterized in terms of antileishmanial activity and metabolic stability. FM09h, with *in vitro* IC_{50} toward amastigotes of less than 1 μM, was further characterized and the *in vivo* efficacy was determined in a CL mouse model.

RESULTS

Design and synthesis of amine-containing flavonoids. To reduce the high molecular weight and metabolic stability problems of FD39, we designed and synthesized 24 amine-containing flavonoids with only one flavone moiety for screening active candidates (Table 1). The compounds were varied with (i) the length and rigidity of the linker and (ii) different R groups attached to terminal amine groups of the linker. As shown in Fig. 1B, linkers R^1 with different length and rigidity were alkylated with 4-chloromethylpyridine to give compounds 1 to 7, which were then subjected to Mitsunobu reaction with 4′-hydroxyflavone to furnish 7 flavonoid monomers (FM01, FM02, FM05, FM13, FM14, FM17, and FM18). FM10 and FM11 were synthesized by reacting 4-hydroxyflavone with compounds 14 or 15 followed by removal of the Boc protecting group. Alkylation of FM10 and FM11 with 4-chloromethylpyridine gave FM12 and FM15, whereas acylation of FM10 with isonicotinoyl chloride in pyridine gave FM16 (Fig. 1B). Coupling of 4-hydroxyflavone with compounds 8 to 11 through the Mitsunobu reaction furnished FM06 and FM24, with intermediates 12 and 13. Then, deprotection of the amine group was done on intermediates 12 and 13 to yield FM20 and FM22 (Fig. 1B). FM20 and FM22 were further coupled with different R^3 and R^4 groups to produce FM03, FM04, FM07, FM08, FM09, FM19, FM21, and FM23.

Of the 24 flavonoid monomers, FM09 was the most potent candidate in killing both promastigotes and amastigotes; however, it was metabolically unstable. Therefore, further structural modification of FM09 was done to seek for better metabolic stability. As

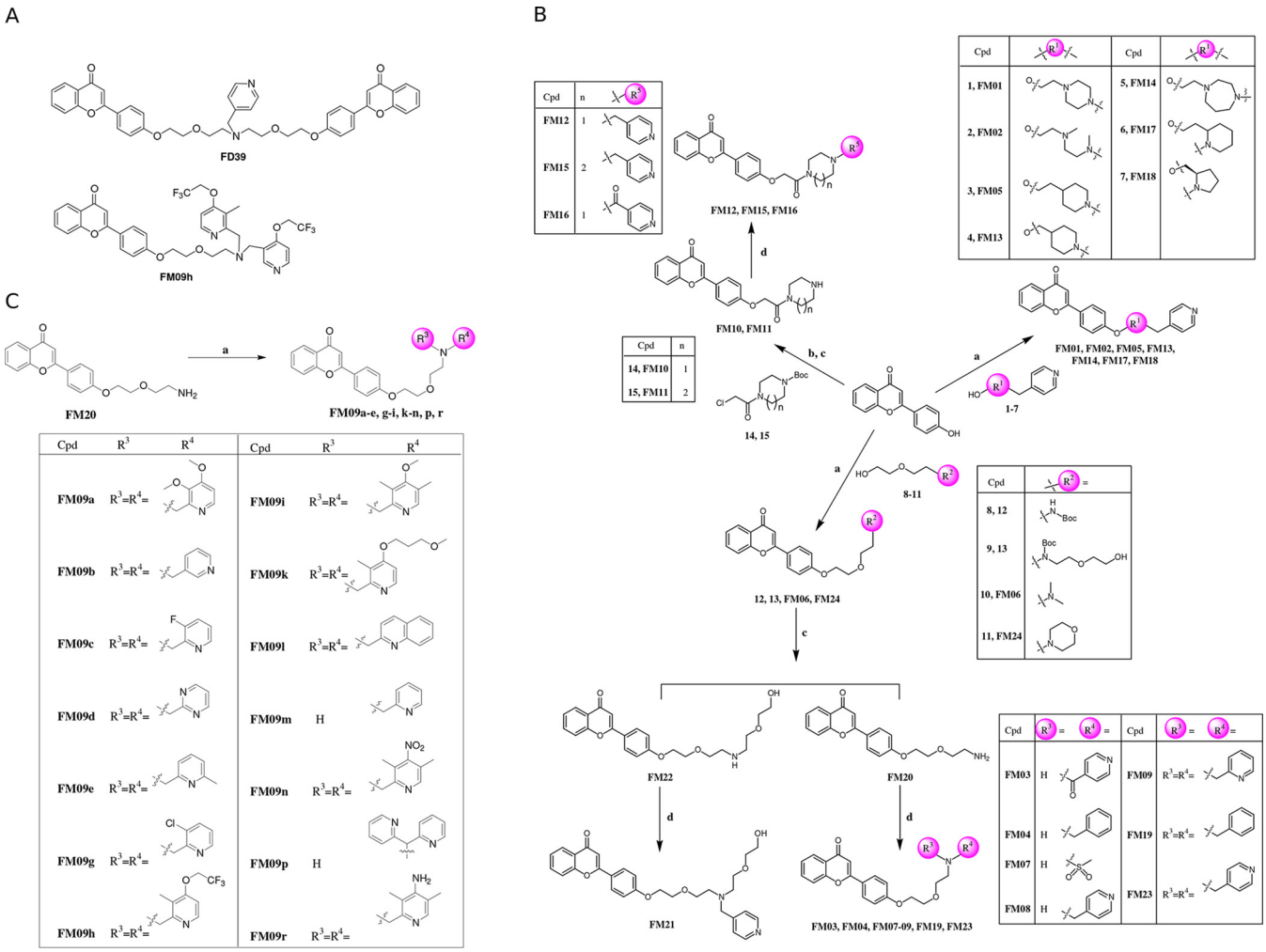


FIG 1 Chemical structure of flavonoid dimer FD39, flavonoid monomer FM09h, and synthetic routes of flavonoid monomers. (A) FD39 was discovered from a group of N-linked flavonoid dimers that was potent against both *Leishmania* promastigotes and *Leishmania* amastigotes (IC_{50} against promastigotes and amastigotes is 0.13 to 0.21 μM and 0.63 μM, respectively) (18). It was also found effective in reducing the lesion in *L. amazonensis*-infected CL model (19). FM09h is reported here. (B) Synthetic route of flavonoid monomers FM01 to 24. Reagents and conditions: (a) PPh_3, THF, DIAD, reflux, 12 h; (b) K_2CO_3, DMF, reflux, 4 h; (c) trifluoroacetic acid, DCM, 0°C to room temperature, 3 h; (d) for compounds FM04, FM08, FM09, FM12, FM13, FM19, and FM23, K_2CO_3, R^3Cl, or R^4Cl, ACN or DMF, reflux, 3 to 4 h; for compound FM03 and FM16, isonicotinoyl chloride, pyridine, 0°C; for FM07, MsCl, NEt_3, DCM, 0°C, 2 h. (C) Synthetic route of FM09 derivatives (FM09a to e, FM09g to i, FM09k to n, FM09p, and FM09r). Reagents and conditions: (a) for compounds FM09a to e, FM09g to i, and FM09k to n, K_2CO_3, R^3Cl, or R^4Cl, ACN or DMF, reflux, 3 to 4 h; for compound FM09p, $MgSO_4$, di(pyridin-2-yl)methanone, DCM, 0°C to room temperature, overnight, N_2 protection, then sodium borohydride, 0°C to room temperature, overnight. For compound FM09r, H_2, Pt/C, room temperature, overnight.

shown in Fig. 1C, modifications were performed by alkylating FM20 with differently substituted pyridyl compounds.

Screening of FM01 to FM24 for their antileishmanial activities and structure-activity relationship. We have tested the *in vitro* potency against two species of cutaneous *Leishmania*, *L. amazonensis* (LV78) and *L. major* (FV1). Many of them (FM01, FM02, FM04, FM05, FM06, FM08, FM09, FM13, FM14, FM15, FM17, FM18, and FM23) were potent and had an IC_{50} of less than 10 μM against promastigotes and amastigotes (Table 1). In general, they were also nontoxic toward mouse L929 fibroblasts (IC_{50} = 13.4 to 95.0 μM) and human macrophage cell line RAW264.7 (IC_{50} = 15.1 to >100 μM) (except for FM23 [IC_{50} toward L929 of 4.7 μM; IC_{50} toward RAW264.7 of 12.4 μM]).

The structure-activity relationships determined in the amine-substituted flavonoids are summarized below.

(i) All the linkers in the present library were attached to the 4′-phenoxy group of the C-ring of the flavone moiety. The linker could be linear (FM02, FM04, FM06, FM08, FM09, FM19, FM20, FM21, FM22, FM23, FM24) or cyclic (FM01, FM05, FM13, FM14, FM17, FM18),

TABLE 1 Antipromastigote and antiamastigote activities of synthetic flavonoid monomers[a]

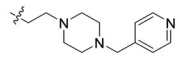
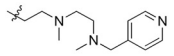
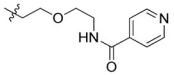
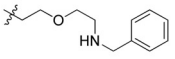
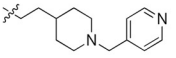
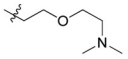
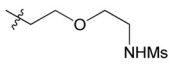
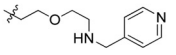
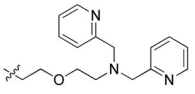
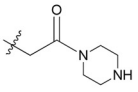
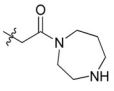
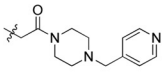
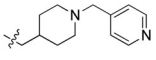
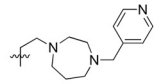
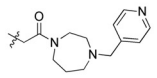
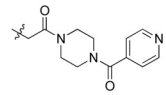
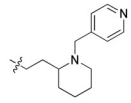
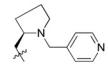
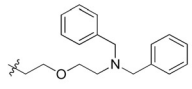
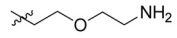
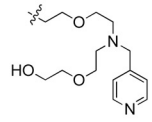
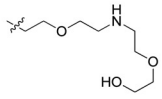
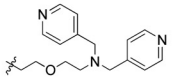
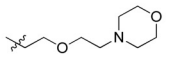
Cpds	R	IC_{50} (μM)				
		Promastigotes		Amastigotes	Mammalian cell lines	
		LV78	FV1	LV78	L929	RAW264.7
FM01		6.0 ± 4.7	5.6 ± 2.7	7.4 ± 1.9	39.7 ± 23.1	64.4 ± 18.8
FM02		4.6 ± 1.7	5.4 ± 2.5	4.7 ± 1.7	23.1 ± 9.6	26.1 ± 5.1
FM03		>50	>50	>10	85.3 ± 27.2	>100
FM04		2.8 ± 2.5	3.8 ± 1.9	3.7 ± 2.9	14.3 ± 10.3	15.1 ± 9.6
FM05		4.0 ± 2.8	3.9 ± 2.2	5.5 ± 3.3	70.1 ± 30.9	37.3 ± 13.7
FM06		2.0 ± 1.0	3.6 ± 1.9	6.3 ± 3.7	16.7 ± 7.3	16.8 ± 5.7
FM07		>50	>50	>10	35.7 ± 28.7	92.2 ± 12.1
FM08		8.3 ± 5.0	9.5 ± 3.1	6.7 ± 2.5	22.1 ± 9.8	49.5 ± 14.8
FM09		1.3 ± 1.1	0.8 ± 0.4	0.4 ± 0.2	16.5 ± 8.9	16.8 ± 5.3
FM10		31.4 ± 7.3	>50	6.0 ± 0.3	30.0 ± 5.8	34.9 ± 4.9
FM11		>50	>50	7.1 ± 2.2	24.3 ± 5.7	23.5 ± 7.6
FM12		>50	>50	>10	73.7 ± 25.1	98.4 ± 4.2
FM13		3.5 ± 3.0	2.7 ± 1.8	5.4 ± 3.9	95.0 ± 15.8	>100

TABLE 1 (Continued)

Cpds	R	IC_{50} (μM)				
		Promastigotes		Amastigotes	Mammalian cell lines	
		LV78	FV1	LV78	L929	RAW264.7
FM14		1.4 ± 1.0	1.6 ± 1.6	4.6 ± 4.3	13.9 ± 8.9	21.6 ± 4.9
FM15		4.8 ± 3.9	6.4 ± 4.7	9.7 ± 0.6	17.2 ± 9.3	47.0 ± 7.3
FM16		>50	>50	>10	87.5 ± 23.1	91.7 ± 20.4
FM17		0.9 ± 0.8	2.1 ± 1.2	4.7 ± 3.4	85.9 ± 29.5	85.1 ± 30.4
FM18		5.5 ± 2.3	4.9 ± 0.9	5.4 ± 4.0	43.2 ± 13.0	55.0 ± 10.7
FM19		13.2 ± 6.7	21.6 ± 8.5	1.3 ± 0.8	82.0 ± 25.9	>100
FM20		14.1 ± 3.7	43.0 ± 16.5	>10	41.8 ± 11.6	20.8 ± 6.4
FM21		8.7 ± 5.1	14.8 ± 11.5	4.2 ± 0.4	13.4 ± 7.0	26.1 ± 3.6
FM22		17.1 ± 3.5	>50	>10	18.6 ± 7.9	10.1 ± 2.0
FM23		3.9 ± 5.4	2.7 ± 1.3	6.5 ± 1.4	4.7 ± 1.0	12.4 ± 1.5
FM24		>50	>50	>10	40.6 ± 30.3	61.7 ± 37.2

[a]Twenty-four novel synthetic flavonoid monomers (FM01 to FM24) were tested for their *in vitro* antipromastigote and antiamastigote activities against *L. amazonensis* (LV78) and *L. major* (FV1). Their cytotoxicity toward mouse fibroblast L929 and mouse macrophage cell line RAW264.7 was also determined as an indicator of general toxicity. All compounds were dissolved in DMSO and the largest amount of DMSO used was 1%, at which no toxicity toward promastigotes, infected macrophages, or mammalian cells was observed. IC_{50} values were presented as mean ± SD. $n = 1$ to 8 independent experiments.

containing carbon, nitrogen, or oxygen, with the amine nitrogen at the end. The amine nitrogen could be 2 (FM18), 3 (FM17), 4 (FM13), or 5 (the remaining FMs) atoms from the 4′-phenoxy attachment (Table 1). These compounds showed similar activities, suggesting that the nature of the linker is not critical in controlling the activity.

(ii) The presence of a free amine group is required. Amide or sulfonamide groups abolished the antileishmanial activities (Table 1). The amine-linked FM01, FM08, and FM14 were more potent than their corresponding amide-linked FM16, FM03, and FM15 by more than 3-fold against promastigotes and more than 2-fold against amastigotes. Amine-linked FM20 (IC_{50} = 14.1 to 43.0 μM) was more active than sulfonamide-linked FM07 ($IC_{50} > 50$ μM) against promastigotes, showing that sulfonamide was not good for activity.

(iii) Pyridylmethyl or benzyl substituent attached to the amine function contributed to the antileishmanial activities. Among FM01 to FM24, 15 of them were active with an IC_{50} of less than 10 μM against LV78 amastigotes. Twelve of them have amino nitrogen substituted with the pyridylmethyl group: FM01, FM02, FM05, FM08, FM09, FM13, FM14, FM15, FM17, FM18, FM21, and FM23. Two of them, FM04 and FM19, have benzyl substituent. FM06, which is a tertiary amine with two methyl substituents, was more potent than the primary amine FM20, the aliphatic secondary amine FM22, or the cyclic aliphatic amine FM24 (Table 1).

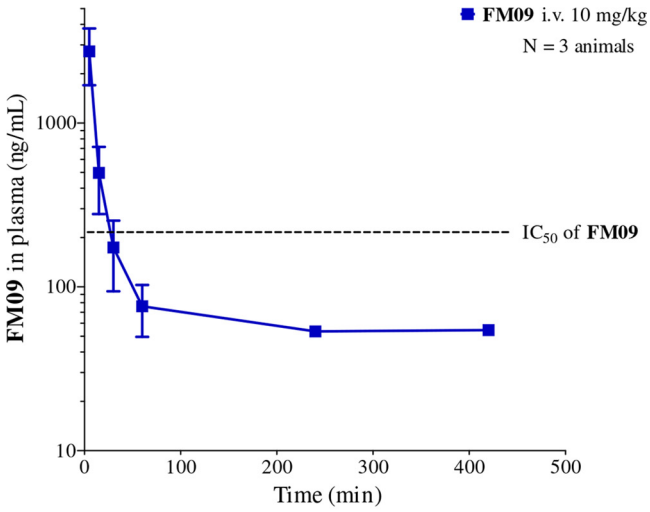
(iv) 2-Pyridylmethyl substituent enhances the antileishmanial potency more effectively than 4-pyridylmethyl substituent. FM09 (IC_{50} = 1.3 μM against promastigotes and IC_{50} = 0.4 μM against amastigotes) was more potent than FM23 (IC_{50} = 3.9 μM against promastigotes and IC_{50} = 6.5 μM against amastigotes) (Table 1).

Of the 24 FM compounds studied, FM09 displayed the strongest antipromastigote and antiamastigote activities toward the cutaneous *Leishmania* (Table 1) because of the amine group in the linker connected with two 2-pyridylmethyl groups that enhanced antileishmanial activities. FM09 also exhibited low toxicity toward L929 mouse fibroblast or RAW264.7 macrophage cell line ($IC_{50} \sim 17$ μM). We therefore chose FM09 for further characterization by studying its pharmacokinetics in mice and metabolic stability.

Pharmacokinetic study of FM09. FM09 was prepared in a hydrochloride salt form and administered intravenously to BALB/c mice at 10 mg/kg. The FM09 level in plasma was quantified by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) (Fig. 2). A high level of FM09 was observed after 5 min with a maximal concentration (C_{max}) of 2,742 ng/ml and an area under the concentration-time curve from 0 to 7 h (AUC_{0-7}) of 63,673 ng-min/ml. The distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$) were 5 and 354 min, respectively (Fig. 2). FM09 metabolites produced by N-dealkylation can be found in plasma (data not shown), suggesting that FM09 may be metabolically labile.

Antileishmanial activity of flavonoid monomers derived from FM09. Because of its metabolic lability, FM09 was modified structurally to seek better metabolic stability while retaining high potency. Fourteen derivatives of FM09 (a to e, g to l, k to n, p, and r) were synthesized according to Fig. 1C. Their antipromastigote and antiamastigote activities are summarized in Table 2. In general, it was found that removal of one of the 2-pyridyl rings in FM09 giving FM09m led to lower potency. Similarly, replacement of the 2-pyridyl rings in FM09 by 3-pyridine (FM09b), pyrimidine (FM09d), or 2-quinoline (FM09l) diminished the activity. On the other hand, any substituent(s) on the 2-pyridyl ring led to no change or slight improvement in potency. The substituent can be electron-donating, such as methyl (FM09e), alkoxy (FM09a, FM09h, FM09i, or FM09k), or amino (FM09r), or electron-withdrawing, such as fluoro (FM09c), chloro (FM09g), or nitro (FM09n). Among the 14 derivatives, FM09a, FM09h, FM09i, and FM09k, all bearing electron-donating substituents, were the most potent ($IC_{50} < 0.5$ μM against promastigotes and IC_{50} = 0.3 μM against amastigotes) (Table 2).

Screening of FM09 derivatives for their metabolic stability. Metabolic stability of these FM09 derivatives was studied by determining the percentage of compounds remaining after incubation with cytochrome P450 (CYP)-rich human or rat liver microsomes for 30 min. These liver microsomes were prepared by ultracentrifugation of human or rat liver homogenates. Among the active compounds derived from FM09, FM09h (human liver microsome [HLM] = 39.4%, rat liver microsome [RLM] = 65.7%) showed reasonable metabolic stability and was more stable compared to the parent compound FM09 (HLM = 2.3%, RLM = 18.1%) (Table 3). FM09d and FM09r were more metabolically stable



	Unit	FM09 i.v. injection
Dose	mg/kg	10
AUC (0-7h)	ng-min/mL	63673
$t_{1/2\,\alpha}$	min	5
$t_{1/2\,\beta}$	min	354
MRT (area)	min	339
Cmax (obs)	ng/mL	2742
CL (obs)	mL/min	157

FIG 2 The semilogarithmic plot of FM09 level in plasma versus time after intravenous injection in mice. FM09 was administered to BALB/c mice intravenously at 10 mg/kg. Animals were sacrificed at different time points (5, 15, 30, 60, 240, and 420 min). Plasma levels of FM09 were quantified by LC-MS/MS. *In vitro* IC_{50} of FM09 (200 ng/ml or 0.4 μM) is indicated as a dashed line. The values are presented as mean ± standard deviation (SD). $n = 3$ animals. Pharmacokinetic parameters were calculated by the pharmacokinetic software Summit PK Solutions. MRT, mean residence time; obs, observed.

but not as active in killing *Leishmania*. Other potent FM09 derivatives with an IC_{50} of less than 10 μM, including FM09, FM09a, FM09c, FM09e, FM09i, FM09k, FM09l, FM09n, and FM09p, were rapidly metabolized (Table 3).

Comparison of FM09h with natural flavonoids and some antileishmanial drugs. We have compared FM09h (structure in Fig. 1A) with other natural flavonoids and marketed antileishmanial drugs. In addition to *L. amazonensis* LV78, we have also tested other CL species, including *L. tropica* EP41, *L. braziliensis* UA847, and *L. major* FV1 and 50122. FM09h was more active than natural flavonoids luteolin and quercetin toward *Leishmania* by more than 18-fold against promastigotes and 8-fold against amastigotes, suggesting a high potency for the synthetic amine-containing flavonoids (Table 4). The potency of FM09h was comparable to or slightly lower than that of amphotericin B (IC_{50} toward promastigotes and amastigotes was 0.13 to 0.34 μM and 0.06 to 0.08 μM, respectively) but was higher than miltefosine by 3- to 59-fold against promastigotes and 3- to 29-fold against amastigotes, higher than paromomycin by >20-fold against promastigotes and >8-fold against amastigotes, higher than pentamidine by 1- to 31-fold against promastigotes and >8-fold against amastigotes, and higher than sodium stibogluconate by at least 15-fold (Table 4). The promising antileishmanial activity of FM09h could be observed microscopically at 0.37 μM. More than 90% of

TABLE 2 Anti-promastigote and antiamastigote activity of FM09 derivatives[a]

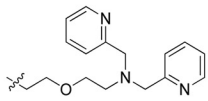
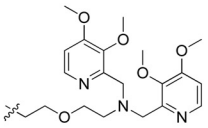
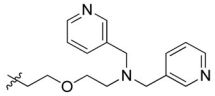
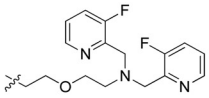
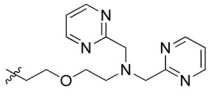
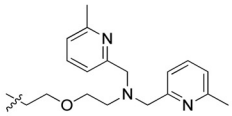
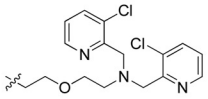
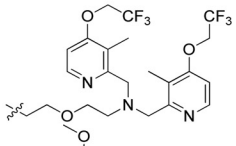
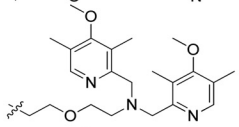
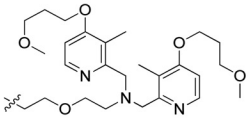
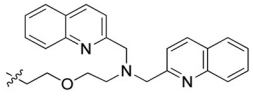
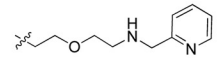
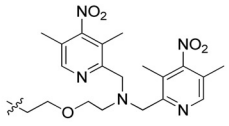
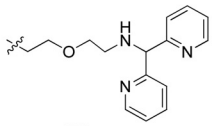
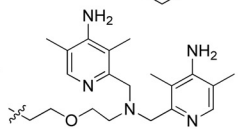
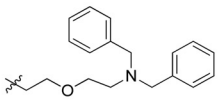
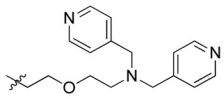
Cpds	R	IC_{50} (μM)				
		Promastigotes	Amastigotes	Mammalian cells		
		LV78	LV78	L929	RAW264.7	PEM
FM09		1.3 ± 1.1	0.4 ± 0.2	16.5 ± 8.9	16.8 ± 5.3	26.9
FM09a		0.3 ± 0.1	0.3 ± 0.1	12.9 ± 3.4	8.5 ± 2.8	10.4 ± 0.6
FM09b		8.1 ± 4.9	>10	75.1 ± 19.2	90.5 ± 0.5	ND
FM09c		3.2 ± 1.3	1.7 ± 0.7	39.0 ± 8.6	39.2 ± 11.9	35.4 ± 1.1
FM09d		35.3 ± 5.9	>10	>100	>100	>100
FM09e		7.4 ± 4.3	2.4 ± 0.6	18.8 ± 1.3	22.1 ± 5.9	ND
FM09g		28.1 ± 12.8	>10	33.7 ± 15.0	34.3 ± 18.9	ND
FM09h		0.5 ± 0.4	0.3 ± 0.1	3.6 ± 0.9	3.8 ± 1.7	6.4
FM09i		0.1 ± 0.0	0.3 ± 0.1	2.3 ± 1.4	3.9 ± 1.1	4.5
FM09k		0.2 ± 0.1	0.3 ± 0.1	4.7 ± 1.2	4.5 ± 2.8	5.3
FM09l		4.4 ± 2.8	3.0 ± 0.4	>100	76.4 ± 20.5	67.1
FM09m		17.6 ± 9.1	4.5 ± 1.4	23.9 ± 1.6	24.7 ± 3.6	28.4
FM09n		4.1 ± 1.9	>10	38.5 ± 7.8	51.9 ± 42.4	ND

TABLE 2 (Continued)

Cpds	R	IC_{50} (μM)				
		Promastigotes	Amastigotes	Mammalian cells		
		LV78	LV78	L929	RAW264.7	PEM
FM09p		3.7 ± 1.3	0.8 ± 0.5	2.1 ± 0.0	6.5	12.5
FM09r		38.3	4.2	73.5 ± 21.7	36.7 ± 8.9	ND
FM19		13.2 ± 6.7	1.3 ± 0.8	82.0 ± 25.9	>100	ND
FM23		3.9 ± 5.4	6.5 ± 2.4	4.7 ± 1.0	12.4 ± 1.5	29.4 ± 2.5

[a]Derivatives of FM09 were tested for their antipromastigote and antiamastigote activities against LV78. Their cytotoxicity toward mouse fibroblast L929, macrophage cell line RAW264.7, and mouse PEM were also measured. All compounds were dissolved in DMSO and the largest amount of DMSO used was 1%, at which no toxicity toward promastigotes, infected macrophages, or mammalian cells was observed. The IC_{50} values were presented as mean ± SD. $n = 1$ to 4 independent experiments. ND, not determined.

intracellular amastigotes (*L. amazonensis* LV78, *L. tropica* EP41, and *L. braziliensis* UA847) inside peritoneal elicited macrophages (PEMs) were eliminated (Fig. 3).

***In vivo* efficacy of FM09h in treating cutaneous leishmaniasis (CL).** CL mouse model was established in 4-week-old female BALB/c mice by intradermal inoculation of *L. amazonensis* promastigotes (See Materials and Methods). Twenty-one days after infection, the mice were randomized into separate groups, including treatment groups, positive control, solvent control, or untreated control. FM09h, dissolved in a formulation of 5% ethanol, 5% Cremophor EL, and 90% saline, was administered by intralesional injection in the treatment group. Intralesional injection of FM09h (10 mg/kg, 8 times every 4 days) inhibited lesion growth as efficiently as that of SSG as positive control (28 mg/kg, 8 times every 4 days) (Fig. 4A and B). FM09h and SSG can reduce the lesion thickness by 72% ± 15% (0.78 ± 0.41 mm, $P < 0.001$) and 63% ± 10% (1.04 ± 0.28 mm, $P < 0.001$), respectively, compared to saline control (2.82 ± 0.58 mm). FM09h showed a dose-dependent efficacy with a greater inhibition of lesion growth (Fig. 4A) and lesion weight (Fig. 4B) in the higher dosage group of 10 mg/kg than in the lower dosage group of 2.5 mg/kg. Throughout the experiment, the body weight of all mice increased steadily (Fig. 4C) and there were no abnormalities in the localized area (Fig. 4D), suggesting that FM09h at indicated dosages did not cause apparent toxicity to the animals. The footpad lesion after the last treatment clearly showed a significant inhibition of lesion growth after intralesional administration of FM09h at 10 mg/kg (Fig. 4D).

DISCUSSION

We have synthesized 38 amine-linked flavonoids and studied their antileishmanial activities. Many of them were found to be active against LV78 promastigotes and amastigotes with an IC_{50} of $<10\,\mu$M. The structure-activity relationships as revealed by the *in vitro* data (Tables 1 and 2) are summarized in Fig. 5. A hydrophobic flavone moiety and a hydrophilic amino moiety are joined at the 4′-phenoxy position of the flavone C-ring by a linker of 2 to 5 atoms composed of carbon, oxygen, or nitrogen. Pyridylmethyl or benzyl substituent attached to the amine function enhanced the antileishmanial activities in the order of

TABLE 3 Metabolic stability of derivatives of FM09[a]

Flavonoid monomer	Percentage of compounds remaining after 30 minutes incubation with:	
	Human liver microsome	Rat liver microsome
FM09	2.3 ± 1.2	18.1 ± 9.3
FM09a	9.0 ± 4.7	9.2 ± 4.5
FM09b	8.7 ± 2.5	7.2 ± 2.2
FM09c	17.5 ± 7.5	17.7 ± 7.4
FM09d	84.6 ± 10.3	83.3 ± 6.4
FM09e	15.5 ± 9.6	21.9 ± 14.8
FM09h	39.4 ± 12.1	65.7 ± 13.1
FM09i	5.3 ± 1.1	14.8 ± 5.0
FM09k	4.4 ± 1.5	10.6 ± 4.2
FM09l	7.8 ± 1.9	16.8 ± 4.2
FM09n	16.5 ± 11.3	13.1 ± 4.0
FM09p	7.4 ± 2.7	19.4 ± 8.5
FM09r	60.5 ± 8.7	70.4 ± 4.7

[a]Derivatives of FM09 were incubated with either human liver microsome (HLM) or rat liver microsome (RLM) for 30 min. Metabolic stability was defined as the percentage of compounds remaining after 30 min of incubation. The values were presented as mean ± SD. $n = 3$ to 6 independent experiments.

2-pyridyl > 4-pyridyl > 3-pyridyl ~ phenyl ~ quinolinyl and pyrimidyl. Substitution in the 2-pyridyl rings with electron-donating groups, such as methyl or alkoxy, rather than electron-withdrawing groups, such as fluoro, chloro, or nitro, appeared to enhance the activity (Tables 1 and 2).

In addition to *in vitro* activity, another consideration for *in vivo* efficacy is the metabolic stability of the compound. Poor metabolic stability implies shorter residence time inside the body and therefore lower concentration of the active compound leading to lower efficacy *in vivo*. For FM09, pharmacokinetics study showed that the $t_{1/2}$ was about 5 min in mice. When administered at 10 mg/kg intravenously (i.v.), the plasma level of FM09 dropped below its *in vitro* IC_{50} toward LV78 (200 ng/ml or 0.4 μM) after 30 min (Fig. 2). After incorporating trifluoroethoxy substituent to the 2-pyridyl rings, FM09h appeared to have improved metabolic stability while maintaining the excellent potency in antileishmanial activity. The use of trifluoroethoxy group to improve the metabolic stability of lipid peroxidation inhibitors (20) and phosphodiesterase 5 (PDE5) inhibitors has been previously reported (21).

FM09h was more potent than other flavonoids against *L. amazonensis*, *L. tropica*, *L. braziliensis*, and *L. major* promastigotes and amastigotes. It was also more potent *in vitro* than most of the current antileishmanial drugs, such as miltefosine, paromomycin, pentamidine, and sodium stibogluconate, and was comparable in potency with amphotericin B (Table 4). The *in vivo* efficacy study demonstrated that FM09h was effective in treating cutaneous leishmaniasis in mice caused by *L. amazonensis*. It is safe for use because the animals did not show any weight loss or apparent toxicity during the treatment (Fig. 4).

Intralesional antimony has been a respected treatment for CL for decades. However, drug resistance has been established because of its widespread misuse. FM09h is more potent than SSG and therefore can be a good replacement to treat CL. In addition, cream application is a preferred approach because of its ease of administration, high cost-effectiveness, fewer adverse effects, and lower risk of developing complications (22). Presently, paromomycin sulfate (15%), in combination with methylbenzethonium chloride (MBCl), is the only clinically available topical treatment and demonstrated a high cure rate in CL-infected patients (23, 24). Other antileishmanial drugs such as amphotericin B (25) and miltefosine (26) were demonstrated to have low skin permeability unless prepared in specific formulations (27). FM09h, with the lipophilic flavone moiety, may well have reasonable permeation through the intercellular lipids around the stratum corneum in skin (28). Future studies including *ex vivo* permeation study and further *in vivo* study to estimate the skin permeability of FM09h should be performed.

TABLE 4 Anti-promastigote and antiamastigote activity of FM09h compared with natural flavonoids and marketed antileishmanial drugs[a]

	IC_{50} (μM)								
	Promastigote					Amastigote			Mammalian cell
Cpds	*L. amazonensis* LV78	*L. tropica* EP41	*L. braziliensis* UA847	*L. major* FV1	*L. major* 50122	*L. amazonensis* LV78	*L. tropica* EP41	*L. braziliensis* UA847	PEM
FM09h	0.5 ± 0.4	1.5 ± 0.6	0.9 ± 0.5	2.1 ± 0.6	1.2 ± 0.3	0.3 ± 0.1	<0.37	<0.37	6.4
Luteolin	>50	>50	>50	>50	>50	>10	>10	>10	>10
Quercetin	>50	>50	>50	>50	>50	>10	>10	>10	>10
Amphotericin B	0.24 ± 0.04	0.13 ± 0.01	0.17 ± 0.04	0.29 ± 0.07	0.34 ± 0.08	0.06 ± 0.04	0.08 ± 0.03	0.05 ± 0.02	7.4 ± 0.4
Miltefosine	29.4 ± 15.8	12.9 ± 2.1	17.8 ± 6.2	5.9 ± 2.4	9.6 ± 5.5	8.7 ± 2.9	4.0 ± 2.3	3.2 ± 2.4	75.3 ± 9.4
Paromomycin	>50	36.2 ± 4.1	19.2 ± 5.1	30.0 ± 5.9	27.1 ± 12.1	>10	>10	>10	>10
Pentamidine	15.7 ± 7.3	14.1 ± 7.6	1.1 ± 0.1	17.8 ± 3.1	7.7 ± 1.4	>10	>10	>10	30.4 ± 10.5
SSG	ND	ND	ND	ND	ND	32.5 ± 24.6	18.5 ± 0.7	35.6 ± 27.9	>11,000

[a]FM09h was compared with natural flavonoids and clinically used antileishmanial drugs, in terms of their activities against promastigotes, amastigotes, and cytotoxicity. All compounds were dissolved in DMSO and the largest amount of DMSO used was 1%, at which no toxicity toward promastigotes, infected macrophages, or mammalian cells was observed. Selective index was calculated as IC_{50} against mammalian cell lines PEM/average IC_{50} against LV78, EP41, and UA847 amastigotes. The IC_{50} values were presented as mean ± SD. $n = 3$ or 4 independent experiments. ND, not determined; Cpds, compounds.

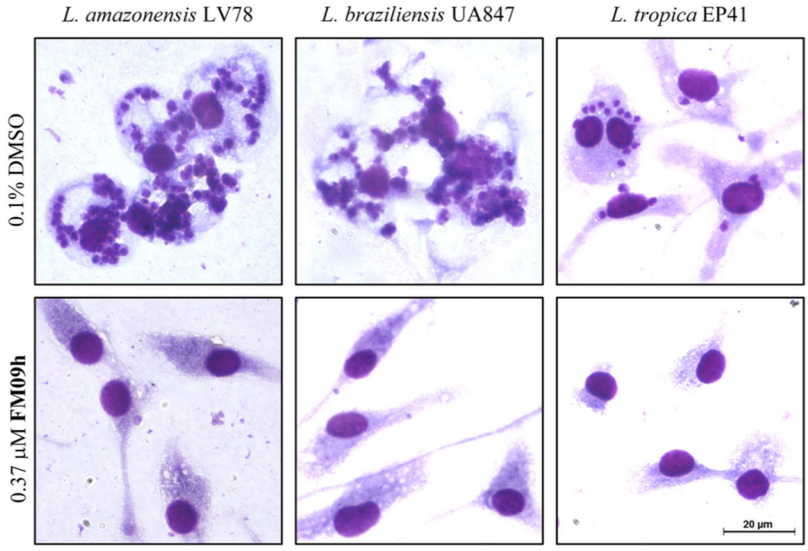


FIG 3 FM09h could eliminate *L. amazonensis*, *L. braziliensis*, and *L. tropica* amastigotes inside the peritoneal elicited macrophages. Peritoneal elicited macrophages (PEMs) infected with *L. amazonensis* LV78, *L. braziliensis* UA847, and *L. tropica* EP41 amastigotes were treated with 0.1% DMSO (top) or 0.37 μM FM09h (bottom). FM09h could eliminate more than 90% of amastigotes inside the macrophages. Magnification at 1,000×.

At this time, the mechanism of action of FM09h against *Leishmania* has not been investigated. Flavonoids such as apigenin (14), quercetin (15), and epigallocatechin-3-gallate (EGCG) (29, 30) have been reported to kill *Leishmania* promastigotes or amastigotes by increasing reactive oxygen species (ROS) level and causing mitochondrial dysfunction. Quercetin and EGCG have also been found to target *Leishmania* arginase, a metallohydrolase responsible for the conversion of arginine to ornithine and urea for the polyamine biosynthesis as well as cell proliferation (31, 32). It has also been reported that luteolin inhibits the topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis (33). To understand the mechanism of action of FM09h, such studies will need to be conducted.

In summary, we discovered that some amine-linked flavonoids were active against cutaneous *Leishmania* parasites at an IC_{50} of ~1 μM *in vitro*. Through structure-activity studies, FM09h was found to show high potency as well as reasonable metabolic stability against human or rat liver microsomes. Intralesional injection of FM09h demonstrated high efficacy in treating cutaneous leishmaniasis in mice caused by *L. amazonensis*. It is safe for use, as the animals did not show any weight loss or apparent toxicity during the treatment.

MATERIALS AND METHODS

Chemical synthesis. Compounds 5, 6, 9, 10, 11, 12,14 to 22, 25 to 34, and 4′-hydroxyflavone were prepared according to reported procedure (16, 17, 34–37). Nuclear magnetic resonance (NMR) spectra were recorded by Bruker MHz DPX400 spectrometer at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C. All NMR measurements were conducted at room temperature, and the reported chemical shifts are displayed as parts per million (ppm) in unit relative to the resonance of $CDCl_3$ (ppm in ^{1}H and ppm for the central line of the triplet in the ^{13}C modes, respectively). Low-resolution and high-resolution mass spectra were obtained on a Micromass (UK) quadruple time of flight (QTOF-2) by electron spray ionization (ESI) mode. Melting points were measured using Electrothermal IA9100 digital melting point apparatus and were uncorrected. All reagents and solvents were reagent grade and were used without further purification unless otherwise stated. The plates used for thin-layer chromatography (TLC) were E. Merck Silica Gel 60F254 (0.25 mm thickness), and they were visualized under short (254 nm) and long (365 nm) UV light. Chromatographic purifications were carried out using MN silica gel 60 (230 to 400 mesh). The purity of tested compounds was determined by high performance liquid chromatography (HPLC), which was performed using an Agilent 1100 series installed with an analytic column of Agilent Prep-Sil Scalar column (4.6 mm by 250 mm, 5 μm) at UV detection of 254 nm (reference at 450 nm) with isocratic elution of acetonitrile (75%)/methanol (25%) at a flow rate of 1 ml/min. All tested compounds were shown to have >95% purity according to HPLC.

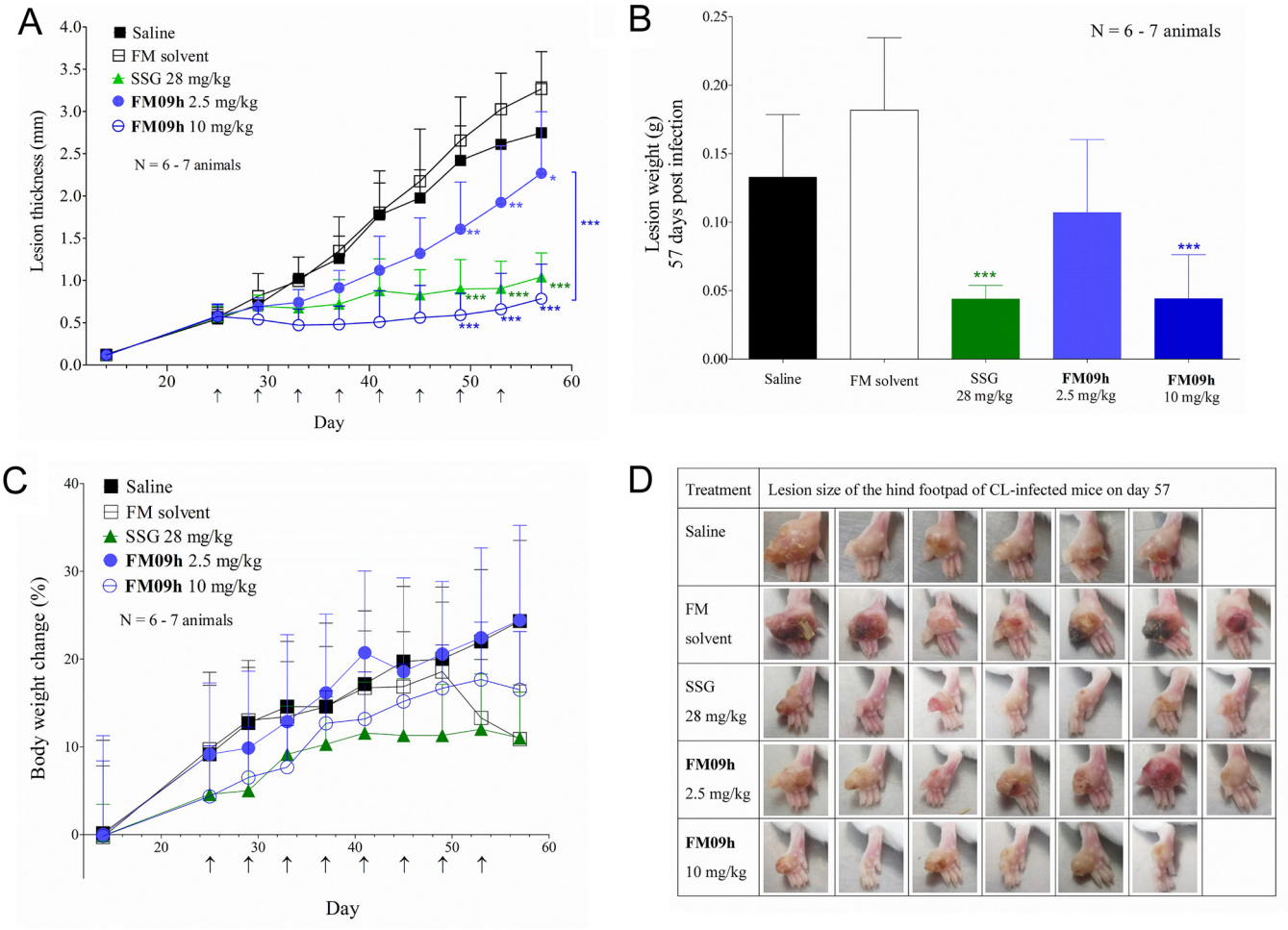


FIG 4 *In vivo* antileishmanial efficacy of FM09h on CL by intralesional injection. BALB/c mice were infected with 1×10^7 stationary-phase *L. amazonensis* LV78 promastigotes in the left hind footpad by intradermal inoculation. Solvent control, positive control (SSG 28 mg/kg), and treatment groups (FM09h 2.5 or 10 mg/kg) were included. (A) Growth rate of lesion. Drugs or saline were administered by intralesional injection once every 4 days for 8 times, starting from day 25. (B) Weight of lesion after 57-day infection. (C) Growth rate of the *L. amazonensis*-infected BALB/c receiving different treatments. (D) Images of footpad lesions of *L. amazonensis*-infected BALB/c mice on the last day of the treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The arrows (↑) indicate the day of drug administration. The values are presented as mean ± SD. $n = 6$ or 7 animals.

General procedure I for the preparation of 12, 13, FM01, FM02, FM05, FM06, FM10, FM11, FM13, FM14, FM17, FM18, and FM24. To a well-stirred mixture of compounds 1 to 11 in Fig. 1B (1 eq), 4′-hydroxyflavone (1 eq), and PPh_3 (1.2 eq) in tetrahydrofuran (THF), DIAD (diisopropyl azodicarboxylate) was added (1.2 eq) dropwise. The reaction mixture was heated to reflux for 12 h. When TLC indicated complete consumption of starting material, the reaction mixture was evaporated under reduced pressure to give a brown oil which was subjected to flash column chromatography on silica gel with gradient elution (20% acetone in dichloromethane [DCM] to 80% acetone to DCM) to furnish the desired product, 12, 13, FM01, FM02, FM05, FM06, FM10, FM11, FM13, FM14, FM17, FM18, and FM24.

General procedure II for the preparation of FM10, FM11, FM20, and FM22. A well-stirred mixture of 4′-hydroxyflavone (1 eq), compound 14 or 15 in Fig. 1B (1.2 eq), and K_2CO_3 (3.0 eq) in dimethylformamide (DMF) (20 ml) was heated to reflux for 3 to 4 h. When TLC indicated complete consumption of starting material, the reaction mixture was poured into water, and the mixture was extracted twice with DCM, dried by $MgSO_4$, filtered, concentrated under reduced pressure, and evaporated under reduced pressure to give brown oil which was subjected to flash column chromatography on silica gel (40% ethyl acetate in *n*-hexane) to furnish the intermediates. One of the intermediates, 12 or 13, was dissolved in DCM at 0°C, and excess trifluoroacetic acid (TFA) was added. The mixture was stirred at room temperature for 3 h. After that, the mixture was poured into a separating funnel containing water, basified to pH 10 with NaOH solution, and extracted with DCM. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated to desired product FM10, FM11, FM20, and FM22.

General procedure III for the preparation of FM04, FM08, FM09, FM12, FM15, FM16, FM19, FM21, FM23, FM09a to e, FM09g to i, and FM09k to n. A well-stirred mixture of FM22 or FM20 (1 eq), R_3X (2.8 eq), and K_2CO_3 (3.0 eq) in acetonitrile (ACN) (20 ml) was heated to reflux for 3 to 4 h. When TLC indicated complete consumption of starting material, the reaction mixture was evaporated under reduced

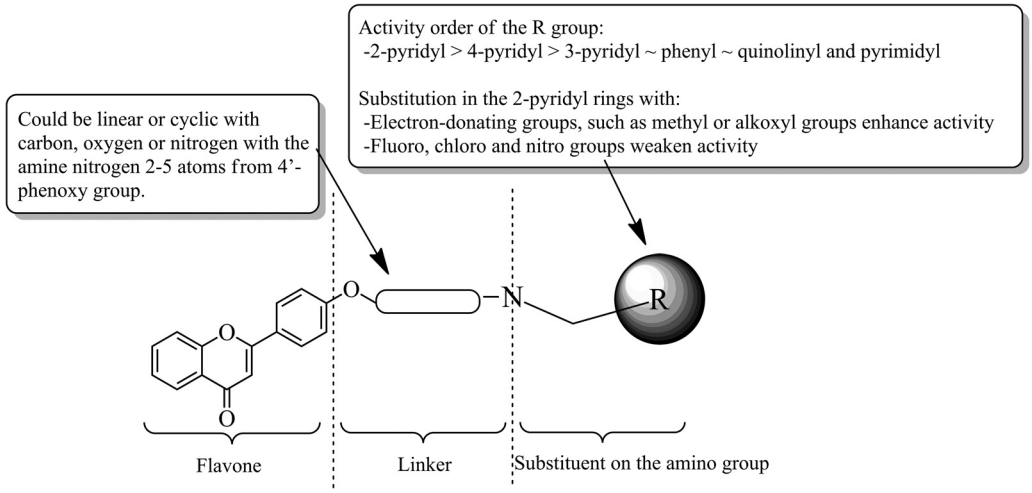


FIG 5 Structure-activity relationship of flavonoid monomers against *Leishmania* promastigotes and amastigotes.

pressure to give brown oil which was subjected to flash column chromatography on silica gel with gradient elution (20% acetone in DCM to 80% acetone in DCM) to furnish the desired product FM04, FM08, FM09, FM19, FM12, FM15, FM16, FM21, FM23, FM09a to e, FM09g to i, and FM09k to n.

Synthesis of 2-(4-(2-(4-(pyridin-4-ylmethyl)piperazin-1-yl)ethoxy)phenyl)-4H-chromen-4-one (FM01). This compound was obtained (0.32 g, 36%) from alcohol 14 (0.45 g, 2.0 mmol), 4′-hydroxyflavone (0.49 g, 2.0 mmol), DIAD (0.43 g, 2.1 mmol), PPh_3 (0.56 g, 2.1 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, J = 5.38 Hz, 2H), 8.08 to 8.11 (m, 1H), 7.73 (d, J = 8.80 Hz, 2H), 7.53 to 7.57 (m, 1H), 7.41 (d, J = 8.31 Hz, 1H), 7.27 (t, J = 7.34 Hz, 1H), 7.18 (d, J = 5.38 Hz, 2H), 6.90 (d, J = 8.80 Hz, 2H), 6.61 (s, 1H), 4.06 (t, J = 5.62 Hz, 2H), 3.41 (s, 2H), 2.75 (t, J = 5.62 Hz, 2H), 2.55 (br. s., 4H), 2.42 (br. s., 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.1, 163.2, 161.5, 156.0, 149.7, 147.5, 133.5, 127.9, 125.5, 125.0, 123.9, 123.8, 123.8, 117.9, 114.9, 106.0, 66.2, 61.6, 56.9, 53.5, 53.0; low-resolution mass spectrometry (LRMS [ESI]) *m/z* 442 (M^+ + H, 100), 464 (M^+ + Na, 10); HRMS (ESI) calculated for $C_{27}H_{28}N_3O_3$ (M^+ + H) 442.2131, Found 442.2138.

Synthesis of 2-(4-(2-(methyl(2-(methyl(pyridin-4-ylmethyl)amino)ethyl)amino)ethoxy)phenyl)-4H-chromen-4-one (FM02). This compound was obtained (0.27 g, 28%) from alcohol 21 (0.49 g, 2.2 mmol), 4′-hydroxyflavone (0.52 g, 2.2 mmol), DIAD (0.45 g, 2.2 mmol), PPh_3 (0.60 g, 2.3 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, J = 5.38 Hz, 2H), 8.17 to 8.23 (m, 1H), 7.85 (d, J = 9.29 Hz, 2H), 7.63 to 7.70 (m, 1H), 7.53 (d, J = 8.80 Hz, 1H), 7.39 (t, J = 7.58 Hz, 1H), 7.21 to 7.28 (m, 2H), 6.99 (d, J = 8.80 Hz, 2H), 6.72 (s, 1H), 4.13 (t, J = 5.62 Hz, 2H), 3.53 (s, 2H), 2.86 (t, J = 5.87 Hz, 2H), 2.63 to 2.70 (m, 2H), 2.52 to 2.58 (m, 2H), 2.36 (s, 3H), 2.24 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.3, 161.6, 156.2, 149.8, 148.4, 133.5, 128.0, 125.6, 125.0, 124.1, 123.9, 123.7, 123.7, 118.0, 115.0, 106.2, 66.5, 61.6, 56.5, 56.0, 55.4, 43.3, 42.8; LRMS (ESI) *m/z* 444 (M^+ + H, 100), 466 (M^+ + Na, 8); HRMS (ESI) calculated for $C_{27}H_{30}N_3O_3$ (M^+ + H) 444.2287, Found 444.2302.

Synthesis of *N*-(2-(2-(4-(4-oxo-4H-chromen-2-yl)phenoxy)ethoxy)ethyl) isonicotinamide (FM03). To a well-stirred solution of FM20 (0.30 g, 0.92 mmol) in pyridine (10 ml) at 0°C, isonicotinoyl chloride (0.20 g, 1.4 mmol) was added at once. The mixture was stirred further for 4 h. After that, the reaction mixture was washed with 1 M HCl solution and saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give a pale brown oil which was subjected to flash column chromatography on silica gel with gradient elution (20% acetone in DCM to 70% acetone in DCM) to furnish the desired product FM03 (0.24 g, 0.56 mmol) in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.72 (d, J = 5.87 Hz, 2H), 8.24 (dd, J = 0.98, 7.82 Hz, 1H), 7.89 (d, J = 9.29 Hz, 2H), 7.67 to 7.75 (m, 1H), 7.54 to 7.65 (m, 3H), 7.44 (t, J = 7.58 Hz, 1H), 7.03 (d, J = 8.80 Hz, 2H), 6.70 to 6.85 (m, 2H), 4.20 to 4.30 (m, 2H), 3.88 to 3.99 (m, 2H), 3.69 to 3.83 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 165.5, 163.2, 161.4, 156.2, 150.6, 141.5, 133.6, 128.1, 125.7, 125.2, 124.6, 124.0, 120.9, 118.0, 115.0, 106.4, 69.7, 69.4, 67.6, 39.8; LRMS (ESI) *m/z* 431 (M^+ + H, 100), 453 (M^+ + Na, 28); HRMS (ESI) calculated for $C_{25}H_{23}N_2O_5$ (M^+ + H) 431.1607, Found 431.1623.

Synthesis of 2-(4-(2-(2-(benzylamino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM04). The title compound FM04 was obtained from FM20 (0.33 g, 1.0 mmol), benzyl bromide (0.25 g, 1.5 mmol), K_2CO_3 (0.20 g, 1.4 mmol), and ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 8.31 Hz, 1H), 7.89 (d, J = 8.80 Hz, 2H), 7.65 to 7.79 (m, 1H), 7.57 (d, J = 8.31 Hz, 1H), 7.43 (t, J = 7.58 Hz, 1H), 7.23 to 7.38 (m, 5H), 7.05 (d, J = 8.80 Hz, 2H), 6.77 (s, 1H), 4.06 to 4.26 (m, 2H), 3.80 to 3.90 (m, 4H), 3.73 (t, J = 5.14 Hz, 2H), 2.88 (t, J = 5.14 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.4, 161.7, 156.2, 140.1, 133.6, 128.4, 128.2, 128.0, 127.0, 125.7, 125.1, 124.3, 124.0, 118.0, 115.1, 106.3, 70.9, 69.4, 67.6, 53.9, 48.7; LRMS (ESI) *m/z* 416 (M^+ + H, 100); HRMS (ESI) calculated for $C_{26}H_{26}NO_4$ (M^+ + H) 416.1862, Found 416.1871.

Synthesis of 2-(4-(2-(1-(pyridin-4-ylmethyl)piperidin-4-yl)ethoxy)phenyl)-4H-chromen-4-one (FM05). This compound was obtained (0.39 g, 36%) from alcohol 16 (0.54 g, 2.5 mmol), 4′-

hydroxyflavone (0.60 g, 2.5 mmol), DIAD (0.55 g, 2.7 mmol), PPh_3 (0.71 g, 2.7 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (d, J = 5.87 Hz, 2H), 8.15 to 8.18 (m, 1H), 7.80 (d, J = 8.80 Hz, 2H), 7.59 to 7.64 (m, 1H), 7.48 (d, J = 8.31 Hz, 1H), 7.34 (t, J = 7.58 Hz, 1H), 7.23 (d, J = 5.87 Hz, 2H), 6.94 (d, J = 8.80 Hz, 2H), 6.68 (s, 1H), 4.02 (t, J = 6.60 Hz, 2H), 3.44 (s, 2H), 2.81 (d, J = 11.25 Hz, 2H), 1.94 to 2.01 (m, 2H), 1.66 to 1.76 (m, 4H), 1.51 (dd, J = 3.91, 7.34 Hz, 1H), 1.27 to 1.37 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.2, 163.3, 161.8, 156.1, 149.7, 149.6, 148.1, 133.5, 127.9, 125.6, 125.0, 123.9, 123.8, 123.7, 117.9, 114.9, 106.0, 65.9, 62.1, 53.9, 35.6, 32.5, 32.3; LRMS (ESI) *m/z* 441 (M^+ + H, 100), 463 (M^+ + Na, 15); HRMS (ESI) calculated for $C_{28}H_{29}N_2O_3$ (M^+ + H) 441.2178, Found 441.2176.

Synthesis of 2-(4-(2-(2-(dimethylamino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM06). This compound was obtained (0.08 g, 9%) from alcohol 3 (0.32 g, 2.4 mmol), 4′-hydroxyflavone (0.60 g, 2.5 mmol), DIAD (0.55 g, 2.7 mmol), PPh_3 (0.71 g, 2.7 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 7.34 Hz, 1H), 7.90 (d, J = 8.80 Hz, 2H), 7.66 to 7.76 (m, 1H), 7.57 (d, J = 8.31 Hz, 1H), 7.43 (t, J = 7.58 Hz, 1H), 7.06 (d, J = 8.80 Hz, 2H), 6.76 (s, 1H), 4.18 to 4.33 (m, 2H), 3.84 to 3.92 (m, 2H), 3.70 (t, J = 5.87 Hz, 2H), 2.59 (t, J = 5.62 Hz, 2H), 2.32 (s, 6×H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.4, 161.7, 156.2, 133.6, 128.0, 125.7, 125.1, 124.2, 124.0, 118.0, 115.1, 106.2, 69.6, 69.4, 67.7, 58.8, 45.8; LRMS (ESI) *m/z* 354 (M^+ + H, 100); HRMS (ESI) calculated for $C_{21}H_{24}NO_4$ (M^+ + H) 354.1705, Found 354.1714.

Synthesis of *N*-(2-(2-(4-(4-oxo-4H-chromen-2-yl)phenoxy)ethoxy)ethyl) methanesulfonamide (FM07). To a well-stirred solution of FM20 (0.13 g, 0.40 mmol) and excess NEt_3 (5 ml) in DCM (5 ml) at 0°C, MsCl (0.10 g, 0.87 mmol) was added dropwise. The reaction mixture was stirred further for 4 h. After that, the reaction mixture was washed with 1 M HCl solution and saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give a pale brown oil which was subjected to flash column chromatography on silica gel with gradient elution (20% acetone in DCM to 70% acetone in DCM) to furnish the desired product FM07 (0.09 g, 0.22 mmol) in 56% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (dd, J = 1.22, 8.07 Hz, 1H), 7.86 (d, J = 8.80 Hz, 2H), 7.65 to 7.71 (m, 1H), 7.54 (d, J = 8.31 Hz, 1H), 7.41 (t, J = 7.34 Hz, 1H), 7.02 (d, J = 8.80 Hz, 2H), 6.74 (s, 1H), 5.13 (t, J = 5.62 Hz, 1H), 4.16 to 4.22 (m, 2H), 3.83 to 3.92 (m, 2H), 3.73 (t, J = 5.14 Hz, 2H), 3.38 (q, J = 5.38 Hz, 2H), 3.00 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.4, 156.2, 133.6, 128.0, 125.6, 125.1, 124.4, 123.9, 118.0, 115.0, 106.2, 70.3, 69.5, 67.5, 43.1, 40.5; LRMS (ESI) *m/z* 404 (M^+ + H, 100), 426 (M^+ + Na, 53); HRMS (ESI) calculated for $C_{20}H_{22}NO_6S$ (M^+ + H) 404.1168, Found 404.1181.

Synthesis of 2-(4-(2-(2-((pyridin-4-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM08). The title compound FM08 (0.04 g, 20%) was obtained from FM20 (0.16 g, 0.49 mmol), 4-chloromethylpyridine hydrochloride (0.19 g, 1.2 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in MeOH (10 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, J = 5.87 Hz, 2H), 8.19 (d, J = 7.82 Hz, 1H), 7.83 (d, J = 8.80 Hz, 2H), 7.63 to 7.68 (m, 1H), 7.52 (d, J = 8.31 Hz, 1H), 7.37 (t, J = 7.58 Hz, 1H), 7.24 (d, J = 5.38 Hz, 2H), 7.00 (d, J = 8.80 Hz, 2H), 6.70 (s, 1H), 4.16 to 4.20 (m, 2H), 3.80 to 3.87 (m, 4H), 3.68 (t, J = 4.89 Hz, 2H), 2.82 (t, J = 5.14 Hz, 2H), 2.08 (br. s., 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.3, 161.6, 156.1, 149.8, 149.3, 133.6, 128.0, 125.6, 125.1, 124.2, 123.9, 122.9, 118.0, 115.0, 106.2, 70.8, 69.4, 67.6, 52.5, 48.7; LRMS (ESI) *m/z* 417 (M^+ + H, 100); HRMS (ESI) calculated for $C_{25}H_{25}N_2O_4$ (M^+ + H) 417.1814, Found 417.1812.

Synthesis of 2-(4-(2-(2-(*bis*(pyridin-2-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09). This compound FM09 (0.12 g, 51%) was obtained from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)pyridine hydrochloride (0.21 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, J = 4.40 Hz, 2H), 8.20 (d, J = 7.82 Hz, 2H), 7.83 (d, J = 8.80 Hz, 2H), 7.51 to 7.68 (m, 6×H), 7.38 (t, J = 7.34 Hz, 1H), 7.09 to 7.13 (m, 2H), 6.99 (d, J = 8.80 Hz, 2H), 6.71 (s, 1H), 4.13 to 4.17 (m, 2H), 3.91 (s, 4H), 3.76 to 3.80 (m, 2H), 3.70 (t, J = 5.87 Hz, 2H), 2.86 (t, J = 5.87 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.3, 161.7, 159.8, 156.2, 149.0, 136.4, 133.6, 127.9, 125.6, 125.1, 124.1, 123.9, 123.0, 121.9, 118.0, 115.1, 106.2, 69.9, 69.2, 67.7, 60.9, 53.6; LRMS (ESI) *m/z* 508 (M^+ + H, 100), 530 (M^+ + Na, 19); HRMS (ESI) calculated for $C_{31}H_{30}N_3O_4$ (M^+ + H) 508.2236, Found 508.2239.

Synthesis of 2-(4-(2-oxo-2-(piperazin-1-yl)ethoxy)phenyl)-4H-chromen-4-one (FM10). This compound was obtained (3.2 g, 82%) from compound 11 (5.0 g, 11 mmol), trifluoroacetic acid (TFA) (10 ml), and DCM (20 ml) according to the general procedure II described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 to 8.17 (m, 1H), 7.78 (d, J = 8.80 Hz, 2H), 7.53 to 7.67 (m, 1H), 7.45 (d, J = 8.31 Hz, 1H), 7.32 (t, J = 7.34 Hz, 1H), 7.00 (d, J = 8.80 Hz, 2H), 6.63 (s, 1H), 4.73 (s, 2H), 3.44 to 3.68 (m, 4H), 2.73 to 2.94 (m, 4H), 1.96 (br. s., 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.2, 165.7, 163.1, 160.7, 156.1, 133.6, 128.0, 125.5, 125.1, 124.8, 123.8, 118.0, 115.1, 106.2, 67.2, 46.5, 46.3, 45.8, 43.2; LRMS (ESI) *m/z* 365 (M^+ + H, 100); HRMS (ESI) calculated for $C_{21}H_{21}N_2O_4$ (M^+ + H) 365.1501, Found 365.1509.

Synthesis of 2-(4-(2-(1,4-diazepan-1-yl)-2-oxoethoxy)phenyl)-4H-chromen-4-one (FM11). This compound was obtained (3.1 g, 79%) from compound 12 (5.0 g, 10 mmol), trifluoroacetic acid (TFA) (10 ml), and DCM (20 ml) according to the general procedure II described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, J = 7.83 Hz, 1H), 7.85 (d, J = 8.80 Hz, 2H), 7.66 (t, J = 7.83 Hz, 1H), 7.51 (d, J = 8.31 Hz, 1H), 7.38 (t, J = 7.58 Hz, 1H), 7.06 (d, J = 8.80 Hz, 2H), 6.71 (s, 1H), 4.79 (d, J = 7.34 Hz, 2H), 3.55 to 3.68 (m, 4H), 2.83 to 3.03 (m, 4H), 2.14 (br. s., 1H), 1.77 to 1.91 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 167.0, 166.9, 163.2, 160.8, 160.8, 156.1, 133.6, 128.1, 125.6, 125.1, 124.8, 123.9, 118.0, 115.2, 106.3, 67.4, 67.2, 50.2, 50.2, 49.2, 48.9, 48.4, 47.6, 46.5, 45.2, 31.1, 29.1; LRMS (ESI) *m/z* 379 (M^+ + H, 100); HRMS (ESI) calculated for $C_{22}H_{23}N_2O_4$ (M^+ + H) 379.1658, Found 379.1672.

Synthesis of 2-(4-(2-oxo-2-(4-(pyridin-4-ylmethyl)piperazin-1-yl)ethoxy)phenyl)-4H-chromen-

4-one (FM12). This compound was obtained (0.30 g, 36%) from FM10 (0.59 g, 1.6 mmol), 4-chloromethylpyridine hydrochloride (0.30 g, 1.8 mmol), and K_2CO_3 (0.60 g) in toluene (30 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, J = 5.38 Hz, 2H), 8.20 to 8.23 (m, 1H), 7.88 (d, J = 8.80 Hz, 2H), 7.66 to 7.71 (m, 1H), 7.54 (d, J = 8.80 Hz, 1H), 7.41 (t, J = 7.58 Hz, 1H), 7.27 (d, J = 5.38 Hz, 2H), 7.08 (d, J = 8.80 Hz, 2H), 6.74 (s, 1H), 4.79 (s, 2H), 3.59 to 3.70 (m, 4H), 3.52 (s, 2H), 2.43 to 2.49 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 165.7, 163.1, 160.6, 156.2, 149.9, 146.9, 133.7, 128.1, 125.7, 125.1, 125.1, 123.9, 123.7, 118.0, 115.2, 106.5, 77.3, 67.5, 61.5, 53.2, 52.8, 45.3, 42.1; LRMS (ESI) *m/z* 456 (M^+ + H, 100); HRMS (ESI) calculated for $C_{27}H_{26}N_3O_4$ (M^+ + H) 456.1923, Found 456.1926.

Synthesis of 2-(4-((1-(pyridin-4-ylmethyl)piperidin-4-yl)methoxy)phenyl)-4H-chromen-4-one (FM13). This compound was obtained (0.32 g, 33%) from alcohol 17 (0.47 g, 2.3 mmol), 4′-hydroxyflavone (0.54 g, 2.3 mmol), DIAD (0.51 g, 2.5 mmol), PPh_3 (0.66 g, 2.5 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, J = 5.38 Hz, 2H), 8.22 (d, J = 7.82 Hz, 1H), 7.87 (d, J = 8.80 Hz, 2H), 7.66 to 7.70 (m, 1H), 7.55 (d, J = 8.31 Hz, 1H), 7.41 (t, J = 7.34 Hz, 1H), 7.29 (d, J = 4.89 Hz, 2H), 7.01 (d, J = 8.80 Hz, 2H), 6.74 (s, 1H), 3.89 (d, J = 5.87 Hz, 2H), 3.52 (s, 2H), 2.91 (d, J = 11.25 Hz, 2H), 2.07 (t, J = 10.76 Hz, 2H), 1.85 (d, J = 8.80 Hz, 3H), 1.41 to 1.52 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.4, 162.0, 156.2, 149.7, 148.1, 133.6, 128.0, 125.7, 125.1, 123.9, 123.9, 123.9, 118.0, 114.9, 106.1, 72.8, 62.1, 53.5, 35.7, 29.1; LRMS (ESI) *m/z* 427 (M^+ + H, 100); HRMS (ESI) calculated for $C_{27}H_{27}N_2O_3$ (M^+ + H) 427.2022, Found 427.2009.

Synthesis of 2-(4-(2-(4-(pyridin-4-ylmethyl)-1,4-diazepan-1-yl)ethoxy)phenyl)-4H-chromen-4-one (FM14). This compound was obtained (0.36 g, 29%) from alcohol 22 (0.64 g, 2.7 mmol), 4′-hydroxyflavone (0.64 g, 2.7 mmol), DIAD (0.61 g, 3.0 mmol), PPh_3 (0.80 g, 3.1 mmol), and THF (30 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, J = 5.38 Hz, 2H), 8.19 (d, J = 7.82 Hz, 1H), 7.84 (d, J = 8.80 Hz, 2H), 7.63 to 7.67 (m, 1H), 7.52 (d, J = 8.31 Hz, 1H), 7.37 (t, J = 7.34 Hz, 1H), 7.26 (d, J = 5.87 Hz, 4H), 7.00 (d, J = 8.80 Hz, 2H), 6.71 (s, 1H), 4.12 (t, J = 5.87 Hz, 2H), 3.58 to 3.69 (m, 2H), 3.00 (t, J = 5.87 Hz, 2H), 2.82 to 2.90 (m, 4H), 2.62 to 2.75 (m, 6×H), 1.74 to 1.85 (m, 2H), 1.20 to 1.25 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.3, 161.7, 156.1, 149.7, 148.9, 133.5, 128.0, 125.6, 125.0, 124.0, 123.9, 123.6, 117.9, 115.0, 106.1, 66.8, 61.7, 56.4, 55.7, 55.3, 54.6, 54.5, 27.8, 22.0; LRMS (ESI) *m/z* 456 (M^+ + H, 100); HRMS (ESI) calculated for $C_{28}H_{30}N_3O_3$ (M^+ + H) 456.2287, Found 456.2277.

Synthesis of 2-(4-(2-oxo-2-(4-(pyridin-4-ylmethyl)-1,4-diazepan-1-yl)ethoxy)phenyl)-4H-chromen-4-one (FM15). This compound was obtained (0.35 g, 41%) from FM11 (0.50 g, 1.3 mmol), 4-chloromethylpyridine hydrochloride (0.30 g, 1.8 mmol), and K_2CO_3 (0.50 g) in MeOH (30 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (dd, J = 5.87, 7.34 Hz, 2H), 8.22 (d, J = 7.82 Hz, 1H), 7.89 (dd, J = 2.93, 8.80 Hz, 2H), 7.67 to 7.72 (m, 1H), 7.55 (d, J = 8.31 Hz, 1H), 7.41 (t, J = 7.58 Hz, 1H), 7.26 (dd, J = 5.62, 9.05 Hz, 3H), 7.09 (dd, J = 5.38, 8.80 Hz, 2H), 6.75 (s, 1H), 4.81 (d, J = 11.74 Hz, 2H), 3.55 to 3.74 (m, 6×H), 2.55 to 2.81 (m, 4H), 1.86 to 1.98 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 167.0, 166.9, 163.1, 160.8, 160.7, 156.2, 149.9, 149.9, 148.1, 133.7, 128.1, 125.7, 125.1, 125.0, 123.9, 123.5, 123.4, 118.0, 115.2, 106.5, 77.3, 67.6, 67.5, 61.5, 61.2, 55.6, 55.3, 54.1, 47.7, 46.4, 46.0, 45.3, 28.7, 27.1; LRMS (ESI) *m/z* 470 (M^+ + H, 100); HRMS (ESI) calculated for $C_{28}H_{28}N_3O_4$ (M^+ + H) 470.2080, Found 470.2063.

Synthesis of 2-(4-(2-(4-isonicotinoylpiperazin-1-yl)-2-oxoethoxy)phenyl)-4H-chromen-4-one (FM16). To a well-stirred solution of FM10 (0.31 g, 0.85 mmol) in pyridine (10 ml) at 0°C, isonicotinoyl chloride (0.20 g, 1.4 mmol) was added at once. The mixture was stirred further for 4 h. After that, the reaction mixture was washed with 1 M HCl solution and saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give a pale brown oil which was subjected to flash column chromatography on silica gel with gradient elution (20% acetone in DCM to 70% acetone in DCM) to furnish the desired product FM16 (0.32 g, 0.68 mmol) in 56% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (d, J = 5.87 Hz, 2H), 8.20 to 8.29 (m, 1H), 7.84 to 8.04 (m, 2H), 7.63 to 7.78 (m, 1H), 7.56 (d, J = 8.31 Hz, 1H), 7.43 (t, J = 7.34 Hz, 1H), 7.30 (s, 2H), 7.10 (br. s., 2H), 6.76 (s, 1H), 4.85 (br. s., 2H), 3.69 to 3.94 (m, 4H), 3.63 (br. s., 2H), 3.40 (br. s., 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 166.2, 162.9, 160.2, 156.2, 150.5, 149.9, 133.7, 128.3, 125.7, 125.5, 125.2, 123.9, 123.4, 121.1, 118.0, 115.1, 106.6, 77.2, 67.8, 60.2, 45.4, 45.1; LRMS (ESI) *m/z* 470 (M^+ + H, 100), 492 (M^+ + Na, 10); HRMS (ESI) calculated for $C_{27}H_{24}N_3O_5$ (M^+ + H) 470.1716, Found 470.1694.

Synthesis of 2-(4-(2-(1-(pyridin-4-ylmethyl)piperidin-2-yl)ethoxy)phenyl)-4H-chromen-4-one (FM17). This compound was obtained (0.34 g, 31%) from alcohol 19 (0.54 g, 2.5 mmol), 4′-hydroxyflavone (0.60 g, 2.5 mmol), DIAD (0.55 g, 2.7 mmol), PPh_3 (0.71 g, 2.7 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (d, J = 5.87 Hz, 2H), 8.12 (dd, J = 0.98, 7.83 Hz, 1H), 7.75 (d, J = 8.80 Hz, 2H), 7.55 to 7.60 (m, 1H), 7.44 (d, J = 8.31 Hz, 1H), 7.29 (t, J = 7.34 Hz, 1H), 7.21 (d, J = 5.38 Hz, 2H), 6.87 (d, J = 8.80 Hz, 2H), 6.63 (s, 1H), 3.97 to 4.07 (m, 2H), 3.88 (d, J = 14.67 Hz, 1H), 3.30 (d, J = 15.16 Hz, 1H), 2.56 to 2.69 (m, 2H), 2.03 to 2.15 (m, 2H), 1.89 to 1.96 (m, 1H), 1.59 to 1.74 (m, 2H), 1.31 to 1.50 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.1, 163.2, 161.7, 156.0, 149.6, 149.4, 133.5, 127.9, 125.5, 125.0, 123.8, 123.7, 123.4, 117.9, 114.8, 106.0, 77.5, 65.4, 57.4, 56.7, 51.1, 30.3, 29.7, 24.5, 22.7; LRMS (ESI) *m/z* 441 (M^+ + H, 100), 463 (M^+ + Na, 21); HRMS (ESI) calculated for $C_{28}H_{29}N_2O_3$ (M^+ + H) 441.2178, Found 441.2166.

Synthesis of (*S*)-2-(4-((1-(pyridin-4-ylmethyl)pyrrolidin-2-yl)methoxy)phenyl)-4H-chromen-4-one (FM18). This compound was obtained (0.34 g, 36%) from alcohol 20 (0.45 g, 2.3 mmol), 4′-hydroxyflavone (0.56 g, 2.4 mmol), DIAD (0.55 g, 2.7 mmol), PPh_3 (0.71 g, 2.7 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 to 8.57 (m, 2H), 8.23 (d, J = 7.82 Hz, 1H), 7.84 to 7.90 (m, 2H), 7.66 to 7.71 (m, 1H), 7.56 (d, J = 8.31 Hz, 1H), 7.41 (t, J = 7.58 Hz, 1H), 7.27 to 7.32 (m, 2H), 6.97 to 7.04 (m, 2H), 6.75 (d, J = 2.93 Hz, 1H), 3.89 to 4.09 (m, 2H), 3.54 to 3.61 (m, 1H), 2.96 to 3.15 (m, 1H), 2.28 to 2.37 (m, 1H), 2.05 to 2.23 (m, 3H), 1.66 to 1.90 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.4, 163.3, 161.7, 160.5, 156.2, 149.8, 149.8, 149.1, 147.5, 133.6, 128.1, 128.0, 125.7, 125.1, 124.1, 124.0, 124.0, 123.7, 123.6, 118.0, 115.9, 114.9, 106.2, 106.2, 77.3,

73.0, 71.9, 62.6, 61.6, 58.8, 57.5, 54.9, 53.3, 29.7, 28.6, 23.3, 23.0; LRMS (ESI) *m/z* 413 (M^+ + H, 100), 435 (M^+ + Na, 11); HRMS (ESI) calculated for $C_{26}H_{25}N_2O_3$ (M^+ + H) 413.1865, Found 413.1870.

Synthesis of 2-(4-(2-(2-(dibenzylamino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM19). The title compound FM19 was obtained from FM20 (0.33 g, 1.0 mmol), benzyl bromide (0.25 g, 1.5 mmol), K_2CO_3 (0.20 g, 1.4 mmol), and ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, J = 6.85 Hz, 1H), 7.88 (d, J = 8.80 Hz, 2H), 7.68 to 7.73 (m, 1H), 7.57 (d, J = 8.31 Hz, 1H), 7.23 to 7.45 (m, 11H), 7.03 (d, J = 8.80 Hz, 2H), 6.77 (s, 1H), 4.16 to 4.21 (m, 2H), 3.76 to 3.84 (m, 2H), 3.66 to 3.72 (m, 6×H), 2.76 (t, J = 6.11 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.4, 161.7, 156.2, 139.8, 133.6, 128.8, 128.2, 128.0, 126.9, 125.7, 125.1, 124.0, 118.0, 115.1, 106.2, 90.6, 70.4, 69.2, 67.7, 59.0, 52.8; LRMS (ESI) *m/z* 506 (M^+ + H, 100); HRMS (ESI) calculated for $C_{33}H_{32}NO_4$ (M^+ + H) 506.2331, Found 506.2332.

Synthesis of 2-(4-(2-(2-aminoethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM20). This compound was obtained (0.7 g, 92%) from compound 4 (1.0 g, 1.95 mmol), trifluoroacetic acid (TFA) (5 ml), and DCM (30 ml) according to the general procedure II described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (dd, J = 1.22, 8.07 Hz, 1H), 7.76 (d, J = 9.29 Hz, 2H), 7.56 to 7.61 (m, 1H), 7.44 (d, J = 8.31 Hz, 1H), 7.31 (t, J = 7.34 Hz, 1H), 6.94 (d, J = 8.80 Hz, 2H), 6.63 (s, 1H), 4.10 to 4.14 (m, 2H), 3.77 to 3.81 (m, 2H), 3.53 (t, J = 5.14 Hz, 2H), 2.84 (t, J = 5.14 Hz, 2H), 1.64 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.2, 163.2, 161.6, 156.0, 133.5, 127.9, 125.5, 125.0, 124.0, 123.8, 117.9, 115.0, 106.0, 73.6, 69.2, 67.6, 41.7; LRMS (ESI) *m/z* 326 (M^+ + H, 100); HRMS (ESI) calculated for $C_{19}H_{20}NO_4$ (M^+ + H) 326.1392, Found 326.1397.

Synthesis of 2-(4-(2-(2-((2-(2-hydroxyethoxy)ethyl)(pyridin-4-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM21). This compound was obtained (0.13 g, 21%) from FM22 (0.50 g, 1.2 mmol), 4-bromomethylpyridine hydrobromide (0.40 g, 1.6 mmol), and K_2CO_3 (0.42 g) in ACN (60 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 to 8.49 (m, 2H), 8.15 (dd, J = 1.47, 7.82 Hz, 1H), 7.77 to 7.84 (m, 2H), 7.59 to 7.66 (m, 1H), 7.45 to 7.53 (m, 1H), 7.34 (dt, J = 1.22, 7.46 Hz, 1H), 7.28 (d, J = 5.87 Hz, 2H), 6.92 to 6.99 (m, 2H), 6.68 (s, 1H), 4.07 to 4.15 (m, 2H), 3.70 to 3.78 (m, 4H), 3.65 to 3.70 (m, 2H), 3.63 (t, J = 5.62 Hz, 2H), 3.55 (t, J = 5.62 Hz, 2H), 3.47 to 3.52 (m, 2H), 2.70 to 2.80 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.3, 161.6, 156.1, 149.5, 149.1, 133.6, 127.9, 125.5, 125.1, 124.1, 123.8, 123.7, 118.0, 115.0, 106.1, 72.3, 69.9, 69.4, 69.3, 67.6, 61.6, 58.7, 54.3, 54.1; LRMS (ESI) *m/z* 505 (M^+ + H, 100), 527 (M^+ + Na, 20); HRMS (ESI) calculated for $C_{29}H_{33}N_2O_6$ (M^+ + H) 505.2339, Found 505.2325.

Synthesis of 2-(4-(2-(2-((2-(2-hydroxyethoxy)ethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM22). This compound was obtained (0.90 g, 93%) from compound 6 (1.2 g, 2.3 mmol), trifluoroacetic acid (TFA) (10 ml), and DCM (30 ml) according to the general procedure II described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, J = 7.2 Hz, 1H), 7.85 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 6.4 Hz, 1H), 7.52 (d, J = 8.0 Hz, 1H), 7.36 (d, J = 6.4 Hz, 1H), 7.00 (d, J = 8.4 Hz, 2H), 6.71 (s, 1H), 4.18 (t, J = 4.2 Hz, 2H), 3.84 (t, J = 4.2 Hz, 2H), 3.55 to 3.71 (m, 8 H), 2.87 to 2.91 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.4, 161.6, 156.2, 133.6, 128.0, 125.7, 125.1, 124.2, 123.9, 118.0, 115.1, 106.2, 72.5, 69.4, 67.6, 61.8, 54.6, 49.1; LRMS (ESI) *m/z* 414.2 (M^+ + H, 100); HRMS (ESI) calculated for $C_{23}H_{27}NO_6$ (M^+ + H) 414.1838, Found 414.1836.

Synthesis of 2-(4-(2-(2-(bis(pyridin-4-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM23). The title compound FM23 (0.039 g, 16%) was obtained from FM20 (0.16 g, 0.49 mmol), 4-chloromethylpyridine hydrochloride (0.19 g, 1.2 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in MeOH (10 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, J = 5.87 Hz, 4H), 8.14 (d, J = 7.82 Hz, 1H), 7.79 (d, J = 8.80 Hz, 2H), 7.60 to 7.62 (m, 1H), 7.59 (t, J = 8.31 Hz, 1H), 7.31 to 7.48 (m, 1H), 7.25 (d, J = 5.87 Hz, 4H), 6.91 (d, J = 8.80 Hz, 2H), 6.66 (s, 1H), 4.09 to 4.11 (m, 2H), 3.71 to 3.73 (m, 2H), 3.60 to 3.63 (m, 4H), 2.67 (t, J = 5.07 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.3, 161.6, 156.2, 152.3, 149.5, 133.6, 127.9, 125.6, 125.1, 124.1, 123.9, 121.8, 118.0, 115.1, 106.2, 69.9, 69.2, 67.8, 59.2, 53.6; LRMS (ESI) *m/z* 508.2 (M^+ + H, 100); HRMS (ESI) calculated for $C_{31}H_{29}N_3O_4$ (M^+ + H) 508.2158, Found 508.2154.

Synthesis of 2-(4-(2-(2-morpholinoethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM24). This compound was obtained (0.09 g, 10%) from alcohol 3 (0.32 g, 2.4 mmol), 4′-hydroxyflavone (0.60 g, 2.5 mmol), DIAD (0.55 g, 2.7 mmol), PPh_3 (0.71 g, 2.7 mmol), and THF (20 ml) according to the general procedure I described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 8.8 Hz, 2H), 7.69 (t, J = 7.2 Hz, 1H), 7.56 (d, J = 8.3 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 7.04 (d, J = 8.8 Hz, 2H), 6.76 (s, 1H), 4.24 to 4.19 (m, 2H), 3.90 to 3.85 (m, 2H), 3.74 (t, J = 4.6 Hz, 6×H), 2.66 (t, J = 5.6 Hz, 2H), 2.55 (s, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.6, 156.2, 133.6, 128.0, 125.7, 125.1, 124.2, 123.9, 118.0, 115.0, 106.2, 77.4, 77.1, 76.7, 69.4, 68.9, 67.6, 66.8, 58.2, 54.0; LRMS (ESI) *m/z* 396 (M^+ + H, 100); HRMS (ESI) calculated for $C_{23}H_{25}NO_5$ (M^+ + H) 396.1733, Found 396.1724.

Synthesis of 2-(4-(2-(2-(bis((3,4-dimethoxypyridin-2-yl)methyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09a). This compound was obtained (0.15 g, 52%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)-3,4-dimethoxypyridine hydrochloride (0.29 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 to 8.14 (m, 3H), 7.81 (d, J = 8.8 Hz, 2H), 7.67 to 7.61 (m, 1H), 7.51 (d, J = 8.3 Hz, 1H), 7.36 (t, J = 7.5 Hz, 1H), 6.97 (d, J = 8.9 Hz, 2H), 6.73 (d, J = 5.5 Hz, 2H), 6.69 (s, 1H), 4.10 to 4.06 (m, 2H), 3.93 (s, 4H), 3.84 (s, 6×H), 3.74 to 3.70 (m, 8H), 3.65 (t, J = 6.2 Hz, 2H), 2.86 (t, J = 6.2 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.7, 159.8, 158.2, 156.2, 140.4, 133.6, 127.9, 125.6, 125.1, 124.1, 123.9, 123.5, 118.0, 115.1, 106.5, 106.2, 69.9, 69.2, 67.7, 60.9, 60.4, 56.1, 53.6; LRMS (ESI) *m/z* 628 (M^+ + H, 100), 650 (M^+ + Na, 11); HRMS (ESI) calculated for $C_{35}H_{37}N_3O_8$ (M^+ + H) 628.2657, Found 628.2651.

Synthesis of 2-(4-(2-(2-(bis(pyridin-3-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09b). This compound was obtained (0.13 g, 53%) from FM20 (0.15 g, 0.46 mmol), 3-(chloromethyl)pyridine hydrochloride (0.21 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml)

according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, J = 4.4 Hz, 2H), 8.20 (d, J = 7.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H), 7.51 to 7.68 (m, 6×H), 7.38 (t, J = 7.3 Hz, 1H), 7.09 to 7.13 (m, 2H), 6.99 (d, J = 8.8 Hz, 2H), 6.71 (s, 1H), 4.13 to 4.17 (m, 2H), 3.86 (s, 4H), 3.76 to 3.80 (m, 2H), 3.70 (t, J = 5.9 Hz, 2H), 2.86 (t, J = 5.9 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.6, 156.1, 151.8, 147.2, 144.4, 136.5, 133.6, 128.0, 125.6, 125.1, 124.1, 123.9, 123.5, 118.0, 115.1, 106.2, 69.9, 69.3, 68.0, 60.9, 53.6; LRMS (ESI) *m/z* 508 (M^+ + H, 100), 530 (M^+ + Na, 9); HRMS (ESI) calculated for $C_{31}H_{30}N_3O_4$ (M^+ + H) 508.2236, Found 508.2238.

Synthesis of 2-(4-(2-(2-(bis((3-fluoropyridin-2-yl)methyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09c). This compound was obtained (0.14 g, 52%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)-3-fluoropyridine hydrochloride (0.24 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, J = 4.6 Hz, 2H), 8.20 (dd, J = 7.9, 1.3 Hz, 1H), 7.83 (d, J = 8.9 Hz, 2H), 7.70 to 7.63 (m, 1H), 7.53 (d, J = 8.3 Hz, 1H), 7.39 (t, J = 7.5 Hz, 1H), 7.31 (dd, J = 13.8, 5.2 Hz, 2H), 7.18 (dt, J = 8.4, 4.3 Hz, 2H), 6.99 (d, J = 8.9 Hz, 2H), 6.71 (s, 1H), 4.15 to 4.11 (m, 2H), 4.05 (s, 2H), 4.04 (s, 2H), 3.80 to 3.76 (m, 2H), 3.71 (t, J = 5.9 Hz, 2H), 2.94 (t, J = 5.9 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.6, 159.8, 156.1, 147.7, 144.1, 133.6, 128.0, 125.6, 125.1, 124.1, 123.9, 123.5, 120.3, 118.0, 115.1, 106.2, 69.8, 69.3, 67.8, 60.8, 53.5; LRMS (ESI) *m/z* 544 (M^+ + H, 100), 566 (M^+ + Na, 15); HRMS (ESI) calculated for $C_{31}H_{27}F_2N_3O_4$ (M^+ + H) 544.2016, Found 544.1998.

Synthesis of 2-(4-(2-(2-(bis(pyrimidin-2-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09d). This compound was obtained (0.13 g, 51%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)pyrimidine hydrochloride (0.21 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (d, J = 4.9 Hz, 4H), 8.17 (dd, J = 7.9, 1.4 Hz, 1H), 7.82 (d, J = 8.9 Hz, 3H), 7.68 to 7.62 (m, 1H), 7.52 (d, J = 8.3 Hz, 1H), 7.37 (t, J = 7.5 Hz, 1H), 7.12 (t, J = 4.9 Hz, 2H), 6.97 (d, J = 8.9 Hz, 2H), 6.70 (s, 1H), 4.25 (s, 4H), 4.13 to 4.10 (m, 2H), 3.78 to 3.75 (m, 2H), 3.58 to 3.56 (m, 2H), 3.07 to 3.05 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 167.8, 163.3, 161.7, 157.2, 156.1, 133.4, 128.0, 125.6, 125.0, 124.1, 123.9, 118.0, 114.6, 113.5, 106.2, 69.7, 69.3, 67.7, 61.2, 53.1; LRMS (ESI) *m/z* 510 (M^+ + H, 100), 532 (M^+ + Na, 16); HRMS (ESI) calculated for $C_{29}H_{27}N_5O_4$ (M^+ + H) 510.2176, Found 510.2169.

Synthesis of 2-(4-(2-(2-(bis((6-methylpyridin-2-yl)methyl)amino)ethoxy)ethoxy) phenyl)-4H-chromen-4-one (FM09e). This compound was obtained (0.13 g, 51%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)-6-methylpyridine hydrochloride (0.23 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 8.5 Hz, 2H), 8.04 (dd, J = 7.9, 1.1 Hz, 1H), 7.98 to 7.94 (m, 3H), 7.76 to 7.70 (m, 3H), 7.54 to 7.45 (m, 3H), 7.05 (d, J = 8.6 Hz, 2H), 6.90 (s, 1H), 4.15 (s, 2H), 4.03 (s, 4H), 3.71 to 3.68 (m, 2H), 3.65 (t, J = 5.6 Hz, 2H), 3.39 (s, 6×H), 2.80 (t, J = 5.6 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 163.2, 161.5, 157.8, 155.9, 156.0, 136.1, 133.4, 128.0, 125.7, 125.1, 124.2, 124.1, 121.5, 120.3, 118.0, 115.1, 106.3, 69.9, 69.0, 67.8, 60.9, 53.8, 26.1; LRMS (ESI) *m/z* 536 (M^+ + H, 100), 558 (M^+ + Na, 11); HRMS (ESI) calculated for $C_{33}H_{33}N_3O_4$ (M^+ + H) 536.2502, Found 536.2509.

Synthesis of 2-(4-(2-(2-(bis((3-chloropyridin-2-yl)methyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09g). This compound was obtained (0.14 g, 51%) from FM20 (0.15 g, 0.46 mmol), 3-chloro-2-(chloromethyl)pyridine hydrochloride (0.26 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (dd, J = 4.6, 1.1 Hz, 2H), 8.19 (dd, J = 7.9, 1.1 Hz, 1H), 7.82 (d, J = 8.8 Hz, 2H), 7.68 to 7.63 (m, 1H), 7.57 (dd, J = 8.0, 1.2 Hz, 2H), 7.52 (d, J = 8.3 Hz, 1H), 7.38 (t, J = 7.5 Hz, 1H), 7.09 (dd, J = 8.0, 4.7 Hz, 2H), 6.98 (d, J = 8.8 Hz, 2H), 6.70 (s, 1H), 4.13 to 4.10 (m, 2H), 4.09 (s, 4H), 3.78 to 3.74 (m, 2H), 3.69 (t, J = 5.9 Hz, 2H), 3.00 (t, J = 5.9 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.7, 159.8, 156.1, 147.2, 134.5, 133.5, 132.1, 128.1, 125.6, 125.1, 124.1, 123.8, 119.3, 118.0, 115.0, 106.2, 69.9, 69.3, 67.7, 60.8, 53.4; LRMS (ESI) *m/z* 576 (M^+ + H, 100), 598 (M^+ + Na, 10); HRMS (ESI) calculated for $C_{31}H_{27}Cl_2N_3O_4$ (M^+ + H) 576.1463, Found 576.1448.

Synthesis of 2-(4-(2-(2-(bis((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methyl)amino)ethoxy) ethoxy)phenyl)-4H-chromen-4-one (FM09h). This compound was obtained (0.18 g, 51%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine hydrochloride (0.36 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, J = 4.6 Hz, 2H), 8.02 (d, J = 7.5 Hz, 1H), 7.67 (d, J = 8.0 Hz, 2H), 7.52 (t, J = 7.3 Hz, 1H), 7.37 (d, J = 8.1 Hz, 1H), 7.23 (t, J = 7.1 Hz, 1H), 6.85 (d, J = 8.1 Hz, 2H), 6.57 (d, J = 4.9 Hz, 2H), 6.52 (s, 1H), 4.33 to 4.23 (m, 4H), 4.00 (s, 2H), 3.75 (s, 4H), 3.60 (d, J = 5.3 Hz, 2H), 3.51 (d, J = 5.3 Hz, 2H), 2.70 (s, 2H), 1.95 (s, 6×H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 165.8, 163.5, 161.6, 160.2, 156.2, 149.4, 133.6, 128.0, 125.6, 125.1, 124.2, 123.8, 123.5, 117.7, 115.1, 109.5, 105.9, 104.2, 83.5, 70.2, 69.5, 67.5, 60.1, 53.9, 11.2; LRMS (ESI) *m/z* 732 (M^+ + H, 100), 754 (M^+ + Na, 23); HRMS (ESI) calculated for $C_{37}H_{35}F_6N_3O_6$ (M^+ + H) 732.2436, Found 732.2421.

Synthesis of 2-(4-(2-(2-(bis((4-methoxy-3,5-dimethylpyridin-2-yl)methyl)amino)ethoxy)ethoxy) phenyl)-4H-chromen-4-one (FM09i). This compound was obtained (0.16 g, 51%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)-4-methoxy-3,5-dimethylpyridine hydrochloride (0.29 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (dd, J = 7.9, 1.3 Hz, 1H), 8.16 (s, 2H), 7.86 (d, J = 8.9 Hz, 2H), 7.72 to 7.67 (m, 1H), 7.56 (d, J = 8.3 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 7.02 (d, J = 8.9 Hz, 2H), 6.74 (s, 1H), 4.16 to 4.12 (m, 2H), 3.81 (s, 3H), 3.73 (t, J = 4.7 Hz, 3H), 3.70 (s, 5H), 3.62 (t, J = 5.6 Hz, 2H), 2.81 (t, J = 5.6 Hz, 2H), 2.22 (s, 6×H), 2.10 (s, 6×H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 165.4, 163.5, 161.6, 158.2, 156.2, 148.0, 133.6, 128.0, 125.6, 125.1, 124.2, 123.8, 117.7, 115.1, 112.5, 106.2, 105.9, 69.0, 68.4, 67.8, 60.8, 60.3, 54.0, 15.2, 12.1; LRMS (ESI) *m/z* 624 (M^+ + H, 100), 646 (M^+ + Na, 7); HRMS (ESI) calculated for $C_{37}H_{41}N_3O_6$ (M^+ + H) 624.3082, Found 624.3086.

Synthesis of 2-(4-(2-(2-(bis((4-(3-methoxypropoxy)-3-methylpyridin-2-yl)methyl)amino) ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09k). This compound was obtained (0.18 g,

51%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)-4-(3-methoxypropoxy)-3-methylpyridine hydrochloride (0.35 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, J = 4.5 Hz, 2H), 8.02 (d, J = 7.6 Hz, 1H), 7.67 (d, J = 8.0 Hz, 2H), 7.52 (t, J = 7.5 Hz, 1H), 7.37 (d, J = 8.1 Hz, 1H), 7.23 (t, J = 7.1 Hz, 1H), 6.85 (d, J = 8.1 Hz, 2H), 6.57 (d, J = 4.9 Hz, 2H), 6.52 (s, 1H), 4.09 to 4.05 (m, 6×H), 3.93 (s, 4H), 3.74 to 3.70 (m, 2H), 3.65 (t, J = 6.2 Hz, 2H), 3.37 to 3.33 (m, 4H), 3.26 (s, 6×H), 2.86 (t, J = 6.2 Hz, 2H), 2.14 to 2.07 (m, 10H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 166.4, 163.5, 161.6, 160.8, 156.2, 147.2, 133.6, 128.0, 125.6, 125.1, 124.2, 123.8, 117.7, 115.1, 113.5, 106.7, 105.9, 72.9, 70.1, 69.5, 66.6, 66.3, 60.2, 59.3, 53.0, 30.2, 11.1; *m/z* 712 (M^+ + H, 100), 734 (M^+ + Na, 15); HRMS (ESI) calculated for $C_{41}H_{49}N_3O_8$ (M^+ + H) 712.3562, Found 712.3557.

Synthesis of 2-(4-(2-(2-(bis(quinolin-2-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09l). This compound was obtained (0.15 g, 51%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)quinoline hydrochloride (0.28 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, J = 7.9 Hz, 1H), 8.03 (t, J = 9.3 Hz, 4H), 7.77 to 7.70 (m, 6×H), 7.65 to 7.61 (m, 3H), 7.50 to 7.42 (m, 3H), 7.36 (t, J = 7.5 Hz, 1H), 6.92 (d, J = 8.6 Hz, 2H), 6.67 (s, 1H), 4.11 (s, 3H), 3.76 to 3.69 (m, 2H), 2.93 (t, J = 5.5 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.4, 163.3, 161.6, 156.1, 155.8, 147.2, 134.8, 133.6, 129.5, 128.7, 128.0, 127.8, 126.3, 125.9, 125.6, 125.1, 124.1, 123.9, 122.5, 118.0, 115.1, 106.2, 69.9, 69.3, 68.0, 60.9, 53.6; LRMS (ESI) *m/z* 608 (M^+ + H, 100), 630 (M^+ + Na, 13); HRMS (ESI) calculated for $C_{39}H_{33}N_3O_4$ (M^+ + H) 608.2509, Found 608.2518.

Synthesis of 2-(4-(2-(2-((pyridin-2-ylmethyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09m). This compound was obtained (0.05 g, 25%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl) pyridine hydrochloride (0.21 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, J = 4.3 Hz, 1H), 8.25 (dd, J = 7.9, 1.4 Hz, 1H), 7.90 (d, J = 8.9 Hz, 2H), 7.74 to 7.68 (m, 1H), 7.65 (td, J = 7.7, 1.7 Hz, 1H), 7.58 (d, J = 8.3 Hz, 1H), 7.46 to 7.41 (m, 1H), 7.33 (d, J = 7.8 Hz, 1H), 7.18 (dd, J = 6.9, 5.3 Hz, 1H), 7.07 (d, J = 8.9 Hz, 2H), 6.77 (s, 1H), 4.26 to 4.22 (m, 2H), 3.98 (s, 2H), 3.91 to 3.87 (m, 2H), 3.75 (t, J = 5.2 Hz, 2H), 2.93 (t, J = 5.2 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.3, 162.7, 161.7, 158.8, 155.6, 149.3, 136.4, 133.6, 127.6, 124.9, 124.5, 124.0, 123.9, 123.1, 121.9, 118.3, 115.7, 106.5, 70.8, 69.5, 68.1, 61.4, 54.0; LRMS (ESI) *m/z* 417 (M^+ + H, 100), 439 (M^+ + Na, 9); HRMS (ESI) calculated for $C_{25}H_{25}N_2O_4$ (M^+ + H) 417.1814, Found 417.1813.

Synthesis of 2-(4-(2-(2-(bis((3,5-dimethyl-4-nitropyridin-2-yl)methyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09n). This compound was obtained (0.05 g, 25%) from FM20 (0.15 g, 0.46 mmol), 2-(chloromethyl)pyridine hydrochloride (0.21 g, 1.3 mmol), and K_2CO_3 (0.20 g, 1.4 mmol) in ACN (20 ml) according to the general procedure III described above. ^{1}H NMR (400 MHz, CD_3OD) δ 8.21 (s, 2H), 8.09 (dd, J = 8.0, 1.5 Hz, 1H), 7.87 (d, J = 8.9 Hz, 2H), 7.82 to 7.75 (m, 1H), 7.65 (d, J = 8.3 Hz, 1H), 7.46 (t, J = 7.2 Hz, 1H), 7.02 (d, J = 8.9 Hz, 2H), 6.71 (s, 1H), 4.22 (dd, J = 5.2, 3.4 Hz, 2H), 3.88 (s, 4H), 3.75 (dd, J = 5.1, 3.4 Hz, 2H), 3.68 (t, J = 4.8 Hz, 2H), 2.90 to 2.86 (m, 2H), 2.12 (s, 6×H), 2.04 (s, 6×H); ^{13}C NMR (101 MHz, CD_3OD) δ 179.0, 164.3, 162.1, 157.9, 157.1, 156.2, 148.0, 134.1, 127.9, 125.2, 124.7, 123.2, 123.1, 122.9, 122.4, 118.0, 114.9, 104.7, 69.3, 69.2, 67.3, 66.8, 59.6, 55.2, 12.4, 11.3. LRMS (ESI) *m/z* 654 (M^+ + H, 100), 676 (M^+ + Na, 3); HRMS (ESI) calculated for $C_{35}H_{35}N_5O_8$ (M^+ + H) 654.2514, Found 654.2516.

Synthesis of 2-(4-(2-(2-((di(pyridin-2-yl)methyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09p). To a well-stirred mixture of FM20 (0.15 g, 0.46 mmol) and $MgSO_4$ (0.20 g, 1.4 mmol) in dry dichloromethane (5 ml) in ice bath, under N_2 protection, di(pyridin-2-yl)methanone (0.08 g, 0.46 mmol) in dry dichloromethane (3 ml) was added dropwise. The mixture was stirred in room temperature overnight. After that, sodium borohydride (0.2g, 5.3 mmol) dissolved in methanol (5 ml) was added dropwise to the reaction mixture in ice bath. The reaction mixture was stirred in room temperature overnight. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give a brown oil which was subjected to flash chromatography on silica gel with gradient elution (20% acetone in DCM to 70% acetone in DCM) to furnish FM09p (0.17 g, 0.35 mmol) in 73% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, J = 4.2 Hz, 2H), 8.22 (dd, J = 7.9, 1.5 Hz, 1H), 7.87 (d, J = 8.9 Hz, 2H), 7.71 to 7.62 (m, 3H), 7.54 (d, J = 8.2 Hz, 1H), 7.44 to 7.37 (m, 3H), 7.19 (dd, J = 6.7, 5.0 Hz, 2H), 7.06 (d, J = 8.9 Hz, 2H), 6.74 (s, 1H), 5.50 (s, 1H), 4.28 to 4.20 (m, 2H), 3.92 to 3.87 (m, 2H), 3.86 to 3.82 (m, 2H), 3.08 (t, J = 5.0 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.2, 163.1, 162.7, 159.1, 156.2, 149.0, 136.4, 132.8, 127.9, 125.6, 125.3, 124.7, 123.9, 123.0, 121.9, 118.0, 115.1, 106.2, 70.2, 68.7, 67.3, 64.8, 52.5; LRMS (ESI) *m/z* 494 (M^+ + H, 100), 516 (M^+ + Na, 22); HRMS (ESI) calculated for $C_{30}H_{27}N_3O_4$ (M^+ + H) 494.2064, Found 494.2071.

Synthesis of 2-(4-(2-(2-(bis((4-amino-3,5-dimethylpyridin-2-yl)methyl)amino)ethoxy)ethoxy)phenyl)-4H-chromen-4-one (FM09r). A well-stirred mixture of FM09n (0.15 g, 0.23 mmol) and 10% Pd/C (0.20 g) in MeOH (10 ml) under H_2 atmosphere was reacted under room temperature overnight. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give a brown oil which was subjected to flash chromatography on silica gel with gradient elution (20% acetone in DCM to 70% acetone in DCM) to furnish FM09r (0.12 g, 0.20 mmol) in 87% yield. ^{1}H NMR (400 MHz, dimethyl sulfoxide [DMSO]-d6) δ 8.07 to 8.02 (m, 3H), 7.82 (t, J = 7.6 Hz, 1H), 7.77 (d, J = 8.3 Hz, 1H), 7.71 (s, 2H), 7.49 (t, J = 7.3 Hz, 1H), 7.11 to 7.08 (m, 2H), 6.94 (s, 1H), 5.32 (s, 4H), 4.11 (m, 2H), 3.60 (m, 2H), 3.54 (s, 4H), 3.46 to 3.42 (m, 2H), 2.55 (t, J = 6.1 Hz, 2H), 2.00 (s, 6×H), 1.91 (s, 6×H); ^{13}C NMR (101 MHz, DMSO-d6) δ 177.4, 163.1, 161.9, 156.1, 154.3, 151.3, 145.9, 134.6, 128.6, 125.8, 125.2, 123.8, 123.7, 118.9, 115.5, 115.2, 115.1, 105.9, 69.2, 68.8, 67.9, 61.0, 56.5, 52.8, 49.1, 15.0, 12.0. LRMS (ESI) *m/z* 593 (M^+ + H, 100); HRMS (ESI) calculated for $C_{35}H_{39}N_5O_4$ (M^+ + H) 593.3028, Found 593.3024.

Chemicals and reagents. Dimethyl sulfoxide (DMSO), Schneider's drosophila medium, phenazine methosulfate (PMS), Giemsa stain, glutamine, pentamidine, amphotericin B, paromomycin, luteolin, and

quercetin were purchased from Sigma-Aldrich. Miltefosine was purchased from Cayman. Sodium stibogluconate (SSG) was a generous gift from GlaxoSmithKline. Gentamicin was purchased from Life Technologies. Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were purchased from Gibco BRL. Fetal bovine serum (FBS) was purchased from HyClone Laboratories. 3-(4,5-Dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) was purchased from Promega. Human liver microsomes (mixed gender human InVitroCYP 150-donor pooled liver microsomes) and rat liver microsomes (male pooled SD rat liver microsome) were purchased from Research Institute for Liver Diseases, RILD (Shanghai, China). Promastigotes of *L. amazonensis* LV78, *L. tropica* EP41, *L. braziliensis* UA847, and *L. major* FV1 were kindly provided by Kwan-Poo Chang from Rosalind Franklin University. Promastigotes of *L. major* 50122 (MHOM/IL/67/JERICHO II [Lm50122]) and mouse fibroblast L929 were purchased from ATCC. Murine macrophages RAW264.7 was a generous gift from Daniel Lee from The Hong Kong Polytechnic University.

Cell culture. *Leishmania* promastigotes were cultured in Schneider's drosophila medium maintained at pH 6.9 and supplemented with 4 mM glutamine, 25 μg/ml gentamicin solution, and 10% (vol/vol) heat-inactivated FBS at 27°C (18, 19). L929 and RAW264.7 were cultured in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

***In vitro* promastigote activity.** A total of 1.5×10^5 promastigote parasites per well were seeded into a 96-well flat-bottom microplate in a final volume of 100 μl medium and incubated with various synthetic flavonoid monomers or reference drugs of different concentrations at 27°C for 72 h. Cell viability was determined using MTS assay previously described (18, 19). Briefly, MTS/PMS mixture (MTS to PMS ratio of 20:1) was added to each well and incubated for 45 min. Absorbance at 490 nm was then measured to calculate the IC_{50}.

***In vitro* amastigote activity.** Mouse peritoneal elicited macrophages (PEM) were obtained as previously described (38). PEMs were resuspended in the supplemented DMEM containing 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. They were seeded into 24-well flat-bottom plate submerged with 12 mm round coverslip at a cell density of 1×10^5 cells per well and allowed to attach to the coverslip overnight. Nonadherent cells as well as red blood cells were washed away with unsupplemented DMEM. Adherent macrophages were infected with late-log promastigotes at parasite to macrophage ratio of 20:1 overnight at 34°C and 5% CO_2. Noninternalized parasites were washed away with unsupplemented DMEM. Infected macrophages were treated with synthetic flavonoid monomers or reference drugs at different concentrations in a total volume of 500 μl of the supplemented DMEM. Infected macrophages were incubated at 37°C with 5% CO_2 for 72 h. After incubation, coverslips with infected macrophages were fixed with methanol and stained with Giemsa for observation under light microscope. Number of amastigotes per 100 macrophages was enumerated to determine IC_{50} (39, 40).

***In vitro* cytotoxicity against mammalian cells.** *In vitro* cytotoxicity of flavonoid monomers toward mouse fibroblast L929, mouse macrophages RAW264.7, and peritoneal elicited macrophages PEM were determined as an indicator of toxicity. These mammalian cells were cultured in supplemented DMEM at 37°C with 5% CO_2. They were seeded at 1×10^4 cells per well in a 96-well flat-bottom microtiter plate with a final volume of 100 μl. They were incubated with different concentrations of synthetic flavonoid monomers or reference drugs at 37°C with 5% CO_2 for 72 h. Cell viability was determined by MTS assay as described above to calculate the IC_{50} for cytotoxicity.

Pharmacokinetic study of FM09. FM09 prepared in a hydrochloride salt form was dissolved in saline and administered intravenously at 10 mg/kg to overnight-starved BALB/c mice ($n = 3$). At different time points (5, 15, 30, 60, 240, and 420 min), blood was collected via cardiac puncture and placed in a heparinized Eppendorf tube. Blood was centrifuged at $16{,}873 \times g$ for 5 min to obtain the plasma. Forty-μl plasma samples were added with 160 μl acetonitrile for protein precipitation followed by centrifugation at $16{,}873 \times g$ for 10 min to pellet precipitated proteins. Supernatant was collected and filtered through a 0.22 μm nylon filter for LC-MS/MS analysis.

Metabolic stability assays. Flavonoid analogs of FM09 (10 μM) dissolved in methanol or acetonitrile ($<$0.2% vol/vol in the reaction) was incubated with 0.1 mg of human or rat liver microsomes in a final volume of 200 μl in a microcentrifuge tube. The reaction mixture was incubated with or without CYP450 cofactor, NADPH (final concentration 2 mM) at 37°C for 30 min (41). After incubation, 200 μl of acetonitrile was added to stop the reaction. Diethyl ether (1.2 ml) was added to extract the flavonoid analogs. Acetonitrile or diethyl ether was removed by evaporation. Remaining flavonoid analogs were reconstituted with 100 μl of acetonitrile for LC-MS analysis.

UPLC/QQQ-MS and UPLC/QTOF-MS analysis protocol. Agilent 6460 ultra performance liquid chromatography-electrospray ionization triple quadrupole mass spectrometer (UPLC-QQQ-MS) and Agilent 6540 UPLC-quadrupole-time of flight mass spectrometer (UPLC-qTOF-MS) was used in sample separation in FM09 detection in plasma and *in vitro* metabolic stability assay, respectively. Waters Acquity UPLC BEH C_{18} and C_8 (1.7 μm, 2.1 by 50 mm) columns were used in stationary phase, while 50 mM ammonium formate (0.1% formic acid, vol/vol) and acetonitrile (0.1% formic acid, vol/vol) were used in the mobile phase. The flow rate was kept at 0.2 ml/min. During separation, a gradient elution protocol was used as follows. Equilibration: 0 to 1 min, 95% ammonium formate. Elution gradient: 1 to 10 min, 5% to 100% acetonitrile. Regeneration: 10 to 12 min, 100% acetonitrile, then 12 to 14 min, 100% to 5% acetonitrile. Reequilibration: 14 to 15 min, 95% ammonium formate. The sample was reconstituted in acetonitrile and kept in the autosampler at 4°C. Ten μl of the sample was injected into MS for analysis. Both mass spectrometers were operated in positive electrospray ionization (ESI+) mode with supply of 300°C gas temperature and sheath gas temperature, 8 liters/min drying gas, 11 liters/min sheath gas flow, and 3.5 kV capillary voltage. To detect FM09, the MS detection in QQQ-MS was set to 508 to 415 at 22 eV

collision voltage. To assess the metabolic stability, the qTOF-MS was set to MS2 scan at *m/z* 100 to 1,000 to detect the analytes.

***In vivo* efficacy CL mouse model by intralesional injection.** Four-week-old female BALB/c mice were maintained under specific pathogen-free conditions prior to *in vivo* efficacy study. *L. amazonensis* promastigotes at stationary phase were resuspended in antibiotic-free DMEM with a ratio of 1×10^7 cells per 50 μl. Such suspension was intradermally inoculated into the left hind footpad using a 30-gauge needle. Twenty-one days after infection (lesion thickness reached approximately 0.5 mm), the mice were randomized into separate groups, including treatment groups, positive control, solvent control, or untreated control. For intralesional injection, the drugs were administered to the lesion in a bolus volume of 50 μl once every 4 days (a total of 8 injections on day 25, 29, 33, 37, 41, 45, 49, and 53). The treatment groups received either 2.5 or 10 mg/kg of flavonoid FM09h dissolved in a formulation of 5% ethanol, 5% Cremophor EL, and 90% saline. The solvent control group received the above-mentioned formulation. The positive control group received 28 mg/kg of SSG dissolved in saline. The untreated control group was given with saline. After the last injection, the animals were monitored for 4 more days before sacrifice on day 57. The size of the lesion was measured every 4 days before drug administration using a digital caliper. The lesion size was determined by subtraction of the thickness of the left lesion-bearing footpad from that of the right uninfected footpad (19). Animal ethics approval has been obtained from the Animal Subjects Ethics Sub-Committee (ASESC) of the Hong Kong Polytechnic University.

Supplemental material. The supplemental material for this article may be found via https://figshare.com/s/7cf40e3251b20c1a203c. HPLC chromatogram of FM09h and ^{1}H NMR and ^{13}C NMR spectra of all representative compounds are listed in Tables 1 and 2.

ACKNOWLEDGMENTS

The work was supported by the Hong Kong Research Grant Council General Research Fund (560813) and the Hong Kong Polytechnic University Project of Strategic Importance (1-ZE22), YZ1Q and G-YBUX. X.L.Z. was supported by the Hong Kong Scholars Program. We thank University Research Facility of Life Sciences (ULS) and University Research Facility in Chemical and Environmental Analysis (UCEA) of The Hong Kong Polytechnic University (PolyU) for providing mass spectrometry equipment. We also thank Centralized Animal Facilities (CAF) of PolyU and the Laboratory Animal Services Centre (LASEC) of The Chinese University of Hong Kong for providing animal pharmacokinetics and efficacy studies, respectively.

REFERENCES

1. World Health Organization. 2010. Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases. WHO Press, Geneva.
2. Singh S, Sivakumar R. 2004. Challenges and new discoveries in the treatment of leishmaniasis. J Infect Chemother 10:307–315. https://doi.org/10.1007/s10156-004-0348-9.
3. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, Kumar PC, Murray HW. 2000. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis 31:1104–1107. https://doi.org/10.1086/318121.
4. Sundar S, Mehta H, Suresh AV, Singh SP, Rai M, Murray HW. 2004. Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations. Clin Infect Dis 38:377–383. https://doi.org/10.1086/380971.
5. Seifert K, Matu S, Javier Pérez-Victoria F, Castanys S, Gamarro F, Croft SL. 2003. Characterisation of Leishmania donovani promastigotes resistant to hexadecylphosphocholine (miltefosine). Int J Antimicrob Agents 22:380–387. https://doi.org/10.1016/s0924-8579(03)00125-0.
6. Chunge CN, Owate J, Pamba HO, Donno L. 1990. Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. Trans R Soc Trop Med Hyg 84:221–225. https://doi.org/10.1016/0035-9203(90)90263-e.
7. Scott JA, Davidson RN, Moody AH, Grant HR, Felmingham D, Scott GM, Olliaro P, Bryceson AD. 1992. Aminosidine (paromomycin) in the treatment of leishmaniasis imported into the United Kingdom. Trans R Soc Trop Med Hyg 86:617–619. https://doi.org/10.1016/0035-9203(92)90151-2.
8. van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M. 2010. Combination therapy for visceral leishmaniasis. Lancet Infect Dis 10:184–194. https://doi.org/10.1016/S1473-3099(10)70011-6.
9. Harborne JB. 1986. Nature, distribution and function of plant flavonoids. Prog Clin Biol Res 213:15–24.
10. Ayaz M, Sadiq A, Junaid M, Ullah F, Ovais M, Ullah I, Ahmed J, Shahid M. 2019. Flavonoids as prospective neuroprotectants and their therapeutic propensity in aging associated neurological disorders. Front Aging Neurosci 11:155. https://doi.org/10.3389/fnagi.2019.00155.
11. Ginwala R, Bhavsar R, Chigbu DI, Jain P, Khan ZK. 2019. Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin. Antioxidants (Basel) 8:35. https://doi.org/10.3390/antiox8020035.
12. Kikuchi H, Yuan B, Hu X, Okazaki M. 2019. Chemopreventive and anticancer activity of flavonoids and its possibility for clinical use by combining with conventional chemotherapeutic agents. Am J Cancer Res 9:1517–1535.
13. Cushnie TP, Lamb AJ. 2005. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 26:343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002.
14. Fonseca-Silva F, Canto-Cavalheiro MM, Menna-Barreto RF, Almeida-Amaral EE. 2015. Effect of apigenin on *Leishmania amazonensis* is associated with reactive oxygen species production followed by mitochondrial dysfunction. J Nat Prod 78:880–884. https://doi.org/10.1021/acs.jnatprod.5b00011.
15. Fonseca-Silva F, Inacio JD, Canto-Cavalheiro MM, Almeida-Amaral EE. 2013. Reactive oxygen species production by quercetin causes the death of *Leishmania amazonensis* intracellular amastigotes. J Nat Prod 76:1505–1508. https://doi.org/10.1021/np400193m.
16. Wong IL, Chan KF, Burkett BA, Zhao Y, Chai Y, Sun H, Chan TH, Chow LM. 2007. Flavonoid dimers as bivalent modulators for pentamidine and sodium stiboglucanate resistance in leishmania. Antimicrob Agents Chemother 51:930–940. https://doi.org/10.1128/AAC.00998-06.
17. Wong IL, Chan KF, Zhao Y, Chan TH, Chow LM. 2009. Quinacrine and a novel apigenin dimer can synergistically increase the pentamidine susceptibility of the protozoan parasite Leishmania. J Antimicrob Chemother 63:1179–1190. https://doi.org/10.1093/jac/dkp130.

18. Wong IL, Chan KF, Chan TH, Chow LM. 2012. Flavonoid dimers as novel, potent antileishmanial agents. J Med Chem 55:8891–8902. https://doi.org/10.1021/jm301172v.
19. Wong IL, Chan KF, Chen YF, Lun ZR, Chan TH, Chow LM. 2014. In vitro and in vivo efficacy of novel flavonoid dimers against cutaneous leishmaniasis. Antimicrob Agents Chemother 58:3379–3388. https://doi.org/10.1128/AAC.02425-13.
20. Irurre J, Jr, Casas J, Ramos I, Messeguer A. 1993. Inhibition of rat liver microsomal lipid peroxidation elicited by 2,2-dimethylchromenes and chromans containing fluorinated moieties resistant to cytochrome P-450 metabolism. Bioorg Med Chem 1:219–225. https://doi.org/10.1016/S0968-0896(00)82124-0.
21. Tollefson MB, Acker BA, Jacobsen EJ, Hughes RO, Walker JK, Fox DN, Palmer MJ, Freeman SK, Yu Y, Bond BR. 2010. 1-(2–(2,2,2-trifluoroethoxy)ethyl-1H-pyrazolo[4,3-d]pyrimidines as potent phosphodiesterase 5 (PDE5) inhibitors. Bioorg Med Chem Lett 20:3125–3128. https://doi.org/10.1016/j.bmcl.2010.03.106.
22. Garnier T, Croft SL. 2002. Topical treatment for cutaneous leishmaniasis. Curr Opin Invest Drugs 3:538–544.
23. El-On J, Livshin R, Even-Paz Z, Hamburger D, Weinrauch L. 1986. Topical treatment of cutaneous leishmaniasis. J Invest Dermatol 87:284–288. https://doi.org/10.1111/1523-1747.ep12696697.
24. Sosa N, Pascale JM, Jiménez AI, Norwood JA, Kreishman-Detrick M, Weina PJ, Lawrence K, McCarthy WF, Adams RC, Scott C, Ransom J, Tang D, Grogl M. 2019. Topical paromomycin for New World cutaneous leishmaniasis. PLoS Negl Trop Dis 13:e0007253. https://doi.org/10.1371/journal.pntd.0007253.
25. Vardy D, Barenholz Y, Cohen R, Zvulunov A, Biton A, Klaus S, Frankenburg S. 1999. Topical amphotericin B for cutaneous leishmaniasis. Arch Dermatol 135:856–857. https://doi.org/10.1001/archderm.135.7.856.
26. Van Bocxlaer K, Yardley V, Murdan S, Croft SL. 2016. Topical formulations of miltefosine for cutaneous leishmaniasis in a BALB/c mouse model. J Pharm Pharmacol 68:862–872. https://doi.org/10.1111/jphp.12548.
27. Van Bocxlaer K, Yardley V, Murdan S, Croft SL. 2016. Drug permeation and barrier damage in Leishmania-infected mouse skin. J Antimicrob Chemother 71:1578–1585. https://doi.org/10.1093/jac/dkw012.
28. Sharadha M, Gowda D, Gupta V, Akhila A. 2020. An overview on topical drug delivery system–updated review. Int J Res Pharm Sci 11:368–385. https://doi.org/10.26452/ijrps.v11i1.1831.
29. Inacio JD, Gervazoni L, Canto-Cavalheiro MM, Almeida-Amaral EE. 2014. The effect of (-)-epigallocatechin 3-O–gallate in vitro and in vivo in Leishmania braziliensis: involvement of reactive oxygen species as a mechanism of action. PLoS Negl Trop Dis 8:e3093. https://doi.org/10.1371/journal.pntd.0003093.
30. Inacio JD, Canto-Cavalheiro MM, Menna-Barreto RF, Almeida-Amaral EE. 2012. Mitochondrial damage contribute to epigallocatechin-3-gallate induced death in Leishmania amazonensis. Exp Parasitol 132:151–155. https://doi.org/10.1016/j.exppara.2012.06.008.
31. da Silva ER, Maquiaveli CC, Magalhães PP. 2012. The leishmanicidal flavonols quercetin and quercitrin target Leishmania (Leishmania) amazonensis arginase. Exp Parasitol 130:183–188. https://doi.org/10.1016/j.exppara.2012.01.015.
32. dos Reis MB, Manjolin LC, Maquiaveli CC, Santos-Filho OA, da Silva ER. 2013. Inhibition of Leishmania (Leishmania) amazonensis and rat arginases by green tea EGCG, (+)-catechin and (-)-epicatechin: a comparative structural analysis of enzyme-inhibitor interactions. PLoS One 8:e78387. https://doi.org/10.1371/journal.pone.0078387.
33. Mittra B, Saha A, Chowdhury AR, Pal C, Mandal S, Mukhopadhyay S, Bandyopadhyay S, Majumder HK. 2000. Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. Mol Med 6:527–541. https://doi.org/10.1007/BF03401792.
34. Chan KF, Zhao Y, Burkett BA, Wong IL, Chow LM, Chan TH. 2006. Flavonoid dimers as bivalent modulators for P-glycoprotein-based multidrug resistance: synthetic apigenin homodimers linked with defined-length poly(ethylene glycol) spacers increase drug retention and enhance chemosensitivity in resistant cancer cells. J Med Chem 49:6742–6759. https://doi.org/10.1021/jm060593+.
35. Chan KF, Zhao Y, Chow TW, Yan CS, Ma DL, Burkett BA, Wong IL, Chow LM, Chan TH. 2009. Flavonoid dimers as bivalent modulators for p-glycoprotein-based multidrug resistance: structure-activity relationships. ChemMedChem 4:594–614. https://doi.org/10.1002/cmdc.200800413.
36. Wong IL, Chan KF, Tsang KH, Lam CY, Zhao Y, Chan TH, Chow LM. 2009. Modulation of multidrug resistance protein 1 (MRP1/ABCC1)-mediated multidrug resistance by bivalent apigenin homodimers and their derivatives. J Med Chem 52:5311–5322. https://doi.org/10.1021/jm900194w.
37. Chan KF, Wong IL, Kan JW, Yan CS, Chow LM, Chan TH. 2012. Amine linked flavonoid dimers as modulators for P-glycoprotein-based multidrug resistance: structure-activity relationship and mechanism of modulation. J Med Chem 55:1999–2014. https://doi.org/10.1021/jm201121b.
38. Zhang X, Goncalves R, Mosser DM. 2008. The isolation and characterization of murine macrophages. Curr Protoc Immunol Chapter 14:Unit 14.1. https://doi.org/10.1002/0471142735.im1401s83.
39. Neal RA, Croft SL. 1984. An in-vitro system for determining the activity of compounds against the intracellular amastigote form of Leishmania donovani. J Antimicrob Chemother 14:463–475. https://doi.org/10.1093/jac/14.5.463.
40. da Luz RI, Vermeersch M, Dujardin JC, Cos P, Maes L. 2009. In vitro sensitivity testing of Leishmania clinical field isolates: preconditioning of promastigotes enhances infectivity for macrophage host cells. AAC 53:5197–5203. https://doi.org/10.1128/AAC.00866-09.
41. Jia L, Liu X. 2007. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr Drug Metab 8:822–829. https://doi.org/10.2174/138920007782798207.