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Development of a Novel Inducer for EBV Lytic Therapy

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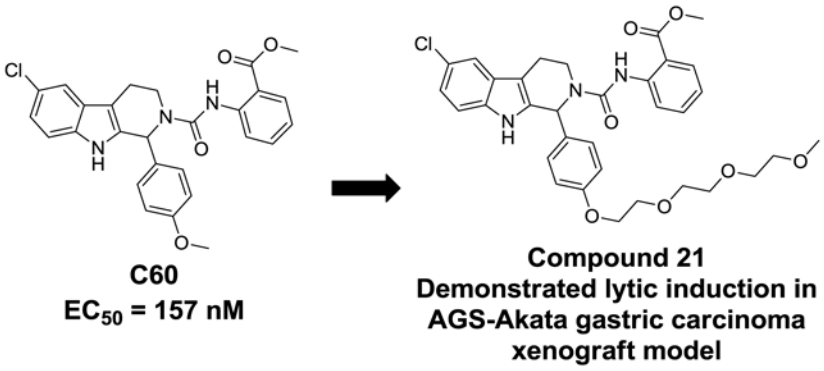
Graphical Abstract



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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The article is the authors' original work and has not received prior publication and is not under consideration for publication elsewhere.

Declaration of interest

Dr. Salvino reports grants from The Wistar Institute, grants from Drexel University, grants from University of Wisconsin-Madison, during the conduct of the study; In addition, Dr. Salvino has a patent WO 2015031759 A1 20150305 issued to The Wistar institute and Drexel University.

Supporting Information

Supplemental Tables, experimental procedures for the synthesis and characterization of the new compounds, and the in vitro activity assay is available free of charge via the Internet.

ANIMAL EXPERIMENTS

The study is compliant with all relevant ethical regulations regarding animal research. For NSG mouse experiments, mice of both sexes were used. Recipient mice were 8-10 weeks old. The University of Wisconsin-Madison is an Accreditation of Laboratory Animal Care (AAALAC) certificated facility. All the procedures and protocols related to animal handling, care, and the treatment in the study were approved by the Institutional Animal Care and Use Committee (IACUC).

Preliminary SAR studies and in vivo reactivation of EBV in SNU719 gastric carcinoma and AGS-Akata gastric carcinoma xenograft mouse model.

Keywords

Epstein-Barr virus; lytic activator; small molecule activator; oncolytic therapy

Epstein-Barr virus (EBV) is a human herpesvirus that infects over 90% of the world's population and is the major cause of infectious mononucleosis.[1,2] Latent infection can drive the formation of Burkitt's lymphoma (BL), Hodgkin lymphomas, nasopharyngeal carcinoma (NPC), and gastric carcinoma (GC).[3-6] In immunosuppressed patients, latent infection with EBV can cause post-transplant lymphoproliferative disease in immunosuppressed patients[7,8] and greatly enhances the risk of developing non-Hodgkin and primary CNS lymphomas in the HIV-positive population.[9,10] Most EBV-associated cancers contain viral DNA that exists predominantly as a latent infection in which only a limited set of viral genes are expressed.[11] These latency associated genes are implicated in host cell proliferation and survival, and latent EBV can directly promote tumor progression. The total number of EBV-associated malignancies is estimated to exceed 200,000 new cancers per year. The near universal presence of EBV in certain tumors suggests that new EBV-targeting therapies could be developed for these malignancies. Current chemotherapeutic treatments of EBV-positive cancers include broad-spectrum cytotoxic drugs that ignore the EBV-positive status of tumors and have limited safety and selectivity. An alternative strategy, referred to as oncolytic therapy, utilizes drugs that stimulate reactivation of latent EBV to enhance the selective killing of EBV-positive tumors, especially in combination with existing inhibitors of herpesvirus lytic replication, like Ganciclovir (GCV).[12-14] This targeted "oncolytic therapy" requires the initiation of the EBV lytic cycle, including expression of viral kinases and DNA polymerase that are exclusively expressed in the lytic phase, but never expressed during the latent phase of infection. This strategy aims to lower side effects associated with standard chemotherapy presently used to treat EBV-positive cancers, and provides a molecular targeted therapeutic strategy by exploiting the biology of EBV as a key etiologic disease factor.

We have previously shown that lytic-inducing agents, such as histone deacetylase (HDAC) inhibitors and DNA damaging agents, can be combined with the FDA approved antiherpesvirus nucleoside analogue, Ganciclovir (GCV), to induce maximal, EBV-dependent tumor cell killing.[15] In cells containing the lytic type of EBV infection, virally encoded kinases (BGLF4 and/or the viral thymidine kinase BXLF4) are expressed which phosphorylate the prodrug, GCV, into a cytotoxic suicide substrate for viral DNA polymerase (BALF5). Phosphorylated GCV inhibits not only the virally encoded DNA polymerase, but also inhibits the host cell DNA polymerase and is thus cytotoxic. Furthermore, phosphorylated GCV can be transferred into nearby cells that are unable to phosphorylate GCV, thus inducing "bystander" killing in EBV positive cells that remain in the latent form of infection.

The major limitation of viral “oncolytic therapy” for EBV cancers is the poor efficiency and nonselectivity of viral reactivation by existing compounds. Of the many known chemical activators of the EBV lytic cycle, only the histone deacetylase inhibitors, derived from butyrate analogues, have been tested in clinical trials.[16,17] In one clinical trial, arginine butyrate was found to be efficacious but was not tolerated due to toxicity, while sodium butyrate (NaB) was found to have unsuitable pharmacokinetics.[16,17] Notably, a pilot study using the HDAC inhibitor, romidepsin, for the treatment of relapsed/refractory extranodal natural killer (NK)/T-cell lymphoma (ENKL) patients in Korea was discontinued due to serious adverse events, due to EBV reactivation in EBV-infected tumor cells.[18] More recent studies have screened clinically approved drugs for potential activators for latent EBV and identified the proteasome inhibitor, bortezomib, as an activator of latent EBV.[19] However, bortezomib induces EBV reactivation only in a small subset of EBV lymphoma types, and requires relatively high doses that produce non-specific cytotoxicity with risk of severe complications.[20] Consequently, the selective and non-toxic induction of EBV lytic reactivation remains an unmet pharmacological need.

Development of new small molecule inducers of EBV lytic reactivation has been limited by incomplete knowledge of the biochemical pathways controlling EBV latency. Histone deacetylase inhibitors, including butyrate-derivatives and trichostatins, have the generic ability to reduce chromatin repression which is known to maintain EBV latency.[21] Other lytic inducers include phorbol esters, calcium ionophores, hypoxia, TNF agonists, and B-cell receptor ligands.[22] Several of these lytic inducers have common and convergent signaling pathways that have been tracked to the transcriptional regulatory elements of the viral immediate early genes, and include transcription factors in the AP1 and MEF2D pathways.[23,24] Several inhibitors of EBV lytic cycle are known, including EBV-encoded latency proteins, LMP1, LMP2, and EBNA1. However, the mechanisms through which these viral proteins restrict lytic cycle gene expression are not completely understood. It is also widely believed that some natural products (e.g. phorbol esters) and environmental co-factors (e.g. malaria surface antigens) stimulate EBV reactivation, but it is not clear how these could be suitable for oncolytic therapy.[25] Consequently, a better understanding of the chemical biology of EBV reactivation is of great biomedical significance for both the development of therapeutic agents for potential oncolytic therapy, and as a probe for biochemical pathways regulating the EBV latent-lytic switch.

Herein we report the preliminary Hit-to-Lead optimization of a new class of small molecules that reactivate latent EBV. A small molecule reactivator of latent EBV in the nanomolar potency range with good preliminary *in vitro* ADME properties has the potential to greatly expand the field of study by providing a useful probe for biological evaluation and target identification, as well as a starting point for a new therapeutic agent for clinical evaluation in synergy with the anti-viral agent GCV. We have previously reported on the **C60** series that was discovered through a high throughput screening campaign.[26] From the 66,840 compounds screened we confirmed the activity of the top five hit compounds through testing in the primary and secondary confirmatory assays as well as re-testing freshly purchased powder samples that were analytically characterized. This provided a small set of closely related urea analogs showing initial potency structure activity relationships (SAR) for the

series (Table 1). Compound **C60** was selected for further follow studies because it showed the greatest potency in MutuI cell based assays (EC_{50} = 157 nM) and consistently stimulated EBV lytic reactivation in multiple cell types.[26] Compound **C60** showed greatly improved potency compared to NaB and TPA which typically require millimolar concentrations to trigger the latent to lytic switch[27,28,29]. It was shown to be an EBV activator that functions synergistically with Ganciclovir (GCV) to selectively kill EBV-positive cells through a distinct mechanism of action from that of the histone deacetylase inhibitor, sodium butyrate, or 12-O-tetradecanoylphorbol-13-acetate (TPA).[25] Importantly, neither **C60** nor GCV alone showed any cytotoxicity up to 30 μM in contrast to NaB, romidepsin, or TPA.

Compound **C60** is a tetrahydro-beta-carboline with structural similarity to tryptoline indole alkaloid natural products which are wide spread in plants and animals. This class of compounds is known for their broad biological activity which includes anti-viral activity, inhibition of VEGF production, and anti-cancer indications.[30] A robust synthesis route was established for the re-synthesis of **C60** as shown in Scheme 1. A Pictet-Spengler reaction using 5-chloro tryptamine (**1**) with p-anisaldehyde (**2**) under acid catalyzed conditions provided the corresponding tetrahydro beta-carboline core structure (**3**), following a slightly modified literature protocol.[31] Reaction with the isocyanate, methyl 2-isocyanato-benzoate, resulted in **C60** as a white solid after chromatographic purification. Testing the re-synthesized material confirmed the original activity. We then evaluated Compound **C60** for its *in vitro* ADME properties. Our initial optimization focused on improving the liabilities in this series which are poor water solubility and a high lipophilicity. Both properties may limit the distribution and *in vivo* pharmacokinetics of the series. The lead showed good stability in liver microsomes, and good plasma stability in addition to potency in the 150-200 nM range in cells.

We then set out to develop additional SAR for the **C60** series to help guide the improvement of the molecular properties while maintaining potency in the cellular assay. Re-examining the results from the high-throughput screening effort identified 199 analogs containing the tetrahydro-beta-carboline core scaffold out of the 66,840 compounds screened. These analogs were cherry picked and then evaluated in a follow-up screen. Using a cell-based luciferase reporter assay for EBV reactivation we tested activity at 2 concentrations (2 and 10 μM). Representative examples are shown in Table 2 for SAR comparison where we looked at percentage of activity compared to Compound **C60** to provide a relative potency for the average values at the 2 μM concentrations. The 199 tetrahydro-β-carboline analogs evaluated in the follow-up HTS campaign had either chlorine (−C1), methoxy (−OMe), or hydrogen (−H) in the 6-position of the A ring. There was a clear preference for the Cl in the 6-position (i.e. **C60** vs **4; C50** vs **6**). Figure 1 shows a summary of the SAR of the 199 available tetrahydro-β-carboline analogs evaluated in this follow-up screen. Para-methoxy substitution on the aromatic ring, **B**, provided potent analogs, other substituents such as a methyl and fluorine were tolerated, however additional analogs are required in order to fully explore the SAR for the **B** ring. Comparison of the substitution pattern for the aromatic urea ring, **C**, suggested that the ortho substitution was preferred (i.e. **C60** vs. **5**; **C50** vs. **7** or **8**). Additional analogs with electron withdrawing and electron donating substitution, and di-substitution on the urea aromatic **C** ring would be helpful to confirm and expand this SAR.

We synthesized additional urea analogs and found that −CN and −C1 are well tolerated in the ortho position of the C ring. Also −C1 is well tolerated in the para position, and only shows a slight loss in activity in the meta position of the C ring.

Although amide linked compounds were contained in the 199 analog follow-up set, all of these compounds contained the non-optimal −H in the 6-position of the indole **A** ring, and were thus weakly active or inactive. Also sulfonamide analogs were not available in this small set. Thus, we synthesized additional **C60** analogs to fill in some of the initial gaps in the SAR focusing on understanding the potency determinates for the series. Representative analogs are shown in Table 3. The synthesis of amide or sulfonamide analogs utilized the 6-chloro-1-(4-methoxyphenyl)-2,3,4,9-tetrahydro-β-carboline intermediate, **3**, (Scheme 1) followed by reaction with either an acid chloride or sulfonyl chloride in the presence of Hunigs base. The scaffold, **3**, was active in the same potency range as **C60** and **C50.** Ortho substitution of the amides is least preferred (i.e. **10**, **13**, **15**), in contrast to what was observed for the urea **C** ring. Para substitution provided amide analogs with comparable potency to **C60**. Meta and para substitution appear to be equally potent (i.e. **11** and **12**). Para methoxy seems best preferred (**11**), with para-Cl substituted analog **9** only slightly less potent, while the electron withdrawing para-trifluormethyl is least preferred (**14**). The two sulfonamide analogs, **16** and **17**, are significantly less potent.

Compound **12** was chosen for ADME profiling to compare against **C60** (Table 4). Water solubility improved and lipophilicity, measured by cLogP, was reduced. Interestingly liver microsomal stability is comparable to **C60**. In an attempt to further improve water solubility for a preliminary in vivo experiment we synthesized three additional analogs, two containing a morpholine group in the meta-position (**18** and **19**) and a phenol analog of **12** (**20**) (Figure 2). These analogs maintained potency, and were more soluble as demonstrated by their improved solubility characteristics when formulated in the 10% DMSO/ 10% solutol/ 80% PBS formulation mixture. We then tested these analogs in an in vivo xenograft model of EBV-associated gastric carcinoma. SNU719 gastric carcinoma cells (1×10^7) were injected subcutaneously into the left and right flanks of NSG mice. After 28 days post-injection, the animals were injected once per day with control vehicle (5% DMSO/ 5% Solutol), PBS, **C60** or **C60** analogues by i.p. administration (30 mg/kg). Animals were euthanized and tumor masses were collected after 4 days of treatment. Tumor masses were homogenized and immunoblot analysis was performed to detect the lytic EBV protein, BZLF1 (Z) and β-actin (loading control). Figure 3 shows that **20** modestly reactivates EBV as demonstrated by the presence of the lytic EBV protein BZLF1 (Z in Fig. 3).

We then synthesized **C60** analogs, **21** and **22,** which have a tri-polyethylene glycol (PEG) moiety attached to the phenolic oxygen (Fig. 4) to further improve water solubility[32] and biodistribution. These are very similar urea analogs (see Fig. 1) to **C60** and the related ortho chloro C-ring analog (not shown) which were consistently showing good activity in the cell based reactivation assay. The main difference was the incorporation of the PEG moiety instead of the methoxy in the B ring (Fig. 1) which significantly improved water solubility, demonstrated by their ease of formulation. We had also shown that the PEG moiety was tolerated and the two analogs had comparable activity to their methoxy substituted counterparts based on Western blot analysis (not shown). We wanted to confirm that

compounds, very similar to **C60,** i.e Compounds **21** and **22** both in the urea series but with improved water solubility, could also reactivate EBV. Therefore these analogs were evaluated in the AGS-Akata gastric carcinoma cell xenograft model. This is a closely related gastric carcinoma model compared to the SNU719 model. The AGS-Akata is a super infected gastric carcinoma cell line routinely used in the Kenney lab.[33] This model would provide analogous preliminary data on the ability of these **C60** analogs to reactivate EBV in another cellular background, thus we choose to use this model to test these more water soluble analogs of **C60**. AGS-Akata cells (1×10^7) were injected into each flank of NSG mice. There are 4 mice per group, i.e. a Compound treated group (**21** or **22**), a Vehicle control group, and a Gemcitabine treated group. After 35 days, mice were injected with 60 mg/kg of Gemcitabine (once), 25 mg/kg of **21** or **22** (once a day for 3 days), and vehicle (5% DMSO and 5% Solutol) (once a day for 3 days). Tumor masses were homogenized and immunoblots were performed to detect the lytic EBV, BZLF1(Z; immediate early protein) and the loading control β-actin. These preliminary in vivo experiment suggest that the **C60** analogs are reactivating latent EBV as demonstrated by the presence of the EBV lytic protein, BZLF1 shown in both animal models. The fact that BZLF1 is not observed from all the animals in the study is mostly due to picking the incorrect section of tumor for Western Blot analysis, or picking a part of the tumor which is not getting the drug delivered adequately to the tumor (perhaps due to poor blood supply to the tumor).

In summary, this new series of reactivators of latent EBV show promise for further hit to lead optimization to provide additional tool compounds for pharmacological evaluation in animal models of lytic therapy in combination with GCV. This series will also provide the basis for chemical biology tool compounds to further uncover the molecular target responsible for the reactivation activity and to further study the mechanism involved in the latent-lytic switch in EBV. Additional analogs and tool compounds will be reported on in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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ABBREVIATIONS

(EBV)	Epstein-Barr virus
(BL)	Burkitt’s lymphoma
(NPC)	nasopharyngeal carcinoma
(GC)	gastric carcinoma
(GCV)	Ganciclovir
(DCM)	dichloromethane

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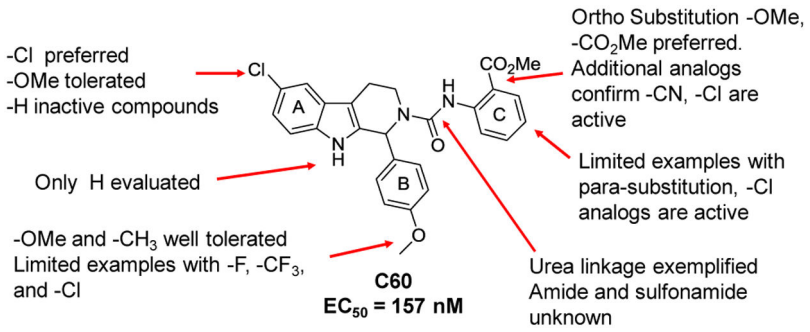
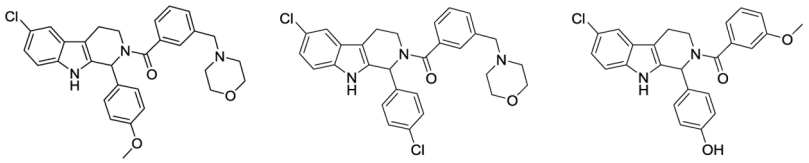


Figure 1.

Summary of the SAR based on 199 analogs available from our screening collection.



18
MW: 516.038
LogP: 5.175
EC_{50}= 277.44 nM

19
MW: 520.454
LogP: 5.091
EC_{50}= 277.442 nM

20
MW: 432.904
LogP: 4.4747
EC_{50}= 310.26 nM

Figure 2.

Analogs of compound **12** with water solubilizing morpholino groups in **18** and **19**, and a phenolic moiety in **20**.

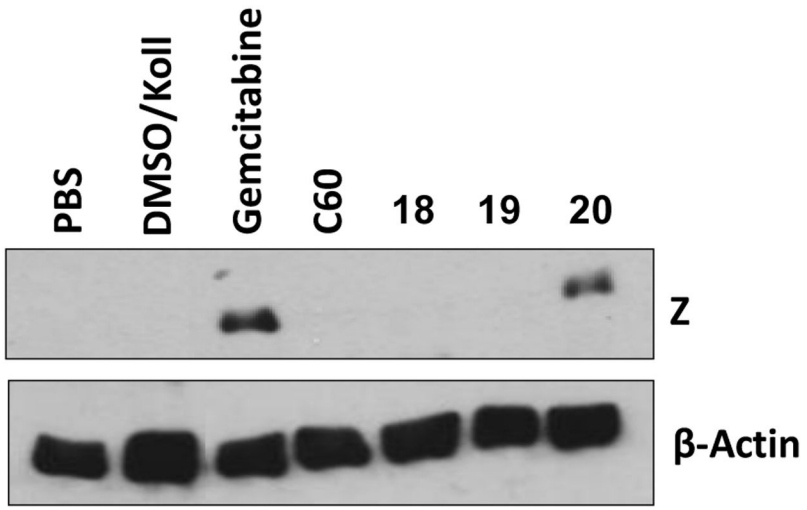


Figure 3.

SNU719 gastric carcinoma tumors were collected after 4 days of treatment with **C60, 18**, **19**, and **20** (30 mg/kg; i.p.). Tumor masses were homogenized and immunoblot analysis was performed to detect the lytic EBV protein, BZLF1 (Z) and β-actin (loading control).

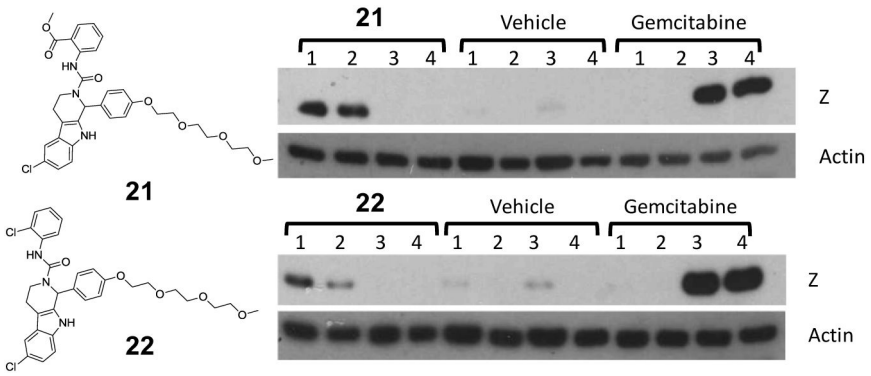
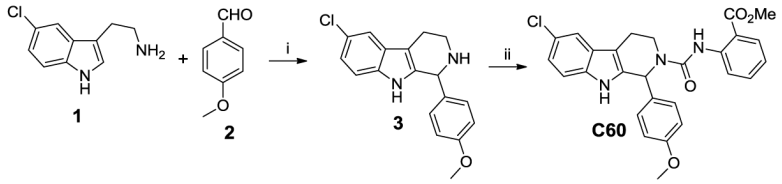


Figure 4.

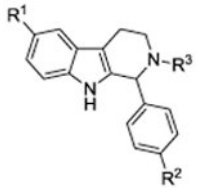
AGS-Akata cells (1×10^7) were injected into each flank of NSG mice. There are 4 mice per group, i.e. a Compound treated group, a Vehicle control group, and a Gemcitabine treated group. After 35 days, mice were injected with 60 mg/kg of Gemcitabine (once), 25 mg/kg of **21** or **22** (once a day for 3 days), and vehicle (5% DMSO and 5% Solutol) (once a day for 3 days). Tumor masses were homogenized and immunoblots were performed to detect the lytic EBV, BZLF1(Z; immediate early protein) and the loading control β-actin.

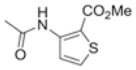
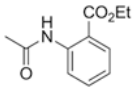
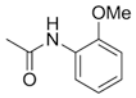
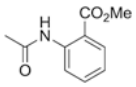
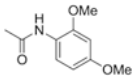


Scheme 1.

Reagents: (i) 0.5 M HCl, reflux 14h. (ii) methyl-2-isocyanato benzoate, DCM, DIEA, rt, 3h.

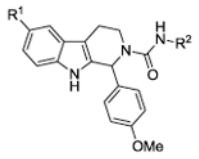
TABLE 1

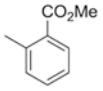
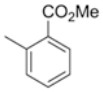
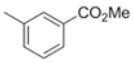
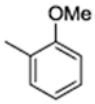
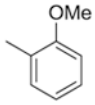
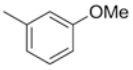
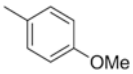


ID	R1	R2	R3	EC_{50} (nM)[a]
C09	−OMe	$-CH_3$		576
C53	−OMe	$-CH_3$		1079
C50	−Cl	−OMe		169
C60	−Cl	−OMe		157
C67	−Cl	−OMe		1128

[a] EC_{50} values represent the average of at least 3 separate experiments.

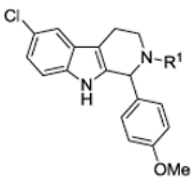
TABLE 2

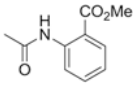
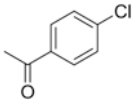
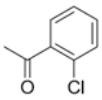
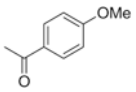
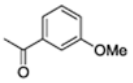
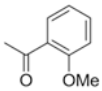
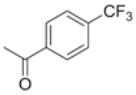
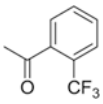
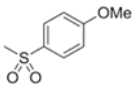
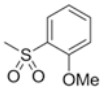


ID	R1	R2	Rel potency[a]
C60	−Cl		100
4	−H		14
5	−Cl		17
C50	−Cl		79
6	−H		15
7	−Cl		43
8	−Cl		31

[a]The relative potency is based on the average of two independent experiments. Rel potency of Cpd= [Cpd % inhib / C60 % inhib] * 100.

TABLE 3



ID	R1	EC_{50} (nM)[a]
C60		157
3	−H	215
9		228
10		2432
11		154
12		168
13		4267
14		434
15		1576
16		8030
17		4008

[a] EC_{50} values represent the average of at least 3 separate experiments.

TABLE 4.

In vitro drug-like properties comparison of C60 and 12

ID	EC_{50}(nM)	Mol Wt	cLogP	% Plasma Protein Binding		Plasma Stability		Liver Microsomal Stability		water Solubility
				Mouse	Human	Mouse	Human	Mouse CLint/ $t_{1/2}$ min	Human CLint/ $t_{1/2}$ min	PBS buffer pH 7.4 µg/mL
IDEALPROBE	<100	<500	2.5-5.0	<95%	<95%	>2h	>2h	<2/ >60	<2/ >60	>500
C60	157	489.95	5.3	NA	NA	>6h	>6h	<0.5/ >90	0.93/ 77.8	<10 (poor)
12	168	446.93	4.7	NA	NA	>6h	>6h	2.3/31	0.67/>90	166