Research at the Interface between Chemistry and Virology: Development of a Molecular Flashlight

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Introduction

Although industrial researchers have been involved in interdisciplinary studies pertaining to virology for several decades, the participation of academic chemists has been significantly increased owing to the importance of HIV (human immunodeficiency virus), the virus which leads to the onset of AIDS (acquired immune deficiency syndrome). The Center for Disease Control estimates that almost 20 million people have been infected by HIV worldwide. The compelling need for effective antiviral therapies for HIV-infected persons, together with rapid advances in understanding the molecular mechanisms of virus replication, has resulted in an explosion of interest in antiviral agents and approaches to antiviral therapies.

This article is organized as follows. After a review of virus replication and current antiviral treatments, we discuss the antiviral activity of the naturally occurring polycyclic quinone hypericin and highlight the necessity of light for its activity. We next focus on how we are using molecular biology to exploit the dependence of light for virucidal activity as a means of specifically targeting hypericin to virus-infected cells. We refer to this targeted light delivery as the "molecular flashlight." Having provided this background, the synthesis of the components of the molecular flashlight are discussed in detail. Finally, on the basis of what we have learned from hypericin and its analogs, we discuss the role of light, on a molecular level, for antiviral activity. In particular, we point out the importance of excited-state intramolecular proton transfer as a primary photophysical process occurring in hypericin upon absorption of a photon. This observation confirms the existence of labile protons in hypericin. Next, on the basis of what is known about the life cycle of the virus, we suggest how the presence of labile protons might contribute to virucidal activity. We also note that hypericin possesses mechanisms of antiviral activity that do not depend on the presence of oxygen. Having established that hypericin has alternate pathways of virucidal activity that do not involve singlet oxygen, we demonstrate that it also executes intermolecular proton transfer and that it is also capable of protonating its surroundings.

Virus Replication and Antiviral Drugs

In general, viruses are classified by structural elements of the virus particle (virion). All viruses have genetic material (DNA or RNA) and some have enzymes necessary for replication packed into a protein shell called a capsid. Many viruses also have a glycoprotein envelope surrounding the capsid. For example, retroviruses (such as HIV), herpes simplex virus (HSV), and influenza virus are all enveloped viruses. Poliovirus, human papilloma virus, and Ebola virus do not have viral envelopes. Viruses replicate intracellularly and rely on host cell machinery for many aspects of replication. The challenge in designing safe, effective antiviral drugs is to specifically interfere with virus replication with minimal deleterious effect to the host cell. Advances in our understanding of molecular mechanisms of virus replication indicate that human pathogenic viruses rely on virus-specific proteins at certain critical points in replication. These proteins include viral enzymes necessary for replication of viral nucleic acid as well as proteins important in regulation of virus gene expression. By identifying the unique structural elements of a particular virus, or unique aspects of virus replication, researchers may begin to rationally design specific inhibitors.

Replication of animal viruses can be viewed as a series of steps, beginning with attachment of the virus to the host cell and concluding with release of infectious virions (an example is shown in Figure 1). The first step in the virus life cycle is entry of the

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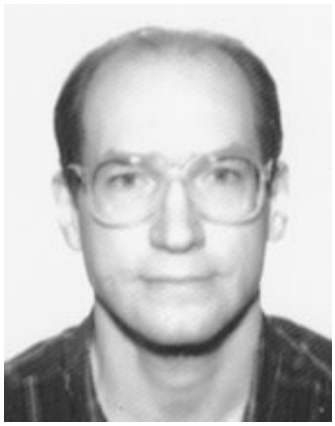

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Susan Carpenter received her Ph.D. in Veterinary and Animal Science from the University of Massachusetts, Amherst, in 1985. She was a staff fellow at the Laboratory for Persistent Viral Diseases, NIAID, NIH, in Hamilton, MT, from 1985 until 1988, when she joined the faculty of the Department of Microbiology, Immunology, and Preventive Medicine at Iowa State University. In 1992, she received the Smith-Kline-Beecham Award for research excellence. Dr. Carpenter's laboratory studies equine infectious anemia virus (EIAV) and bovine immunodeficiency virus (BIV) as animal lentivirus models of HIV infection and AIDS. The major research emphasis is centered on defining virus–host interactions important in viral persistence and pathogenesis. An additional area of investigation is the evaluation and characterization of the antiviral properties of hypericin, a naturally occurring compound which exhibits potent antiviral and antitumor activity in the presence of light. Dr. Carpenter and her collaborators are particularly interested in elucidating the mechanisms of the light-activated antiviral activity of hypericin and in developing novel strategies using chemiluminescence for photoactivation of hypericin in vivo.

virion into a host cell. Initially, the virus interacts with a receptor on the surface of the host cell and penetrates the host cell membrane. In some cases, the cell membrane may engulf the virion (a process called endocytosis), or viral entry may proceed by direct fusion with the host cell membrane. Once inside the cell, the virus uncoats and the viral nucleic acid is released into the cell. In many cases, regulatory proteins are transcribed and translated prior to synthesis of viral nucleic acid and viral structural proteins. Eventually, assembly of the intact virion takes place and the newly generated virions are released from the host cell by two processes. In a process called budding, the virion passes through the host cell membrane without disruption of the cell. In the other process, the cell bursts, releasing virus particles all at once. Once the new virions are released, they then infect other host cells.

Any of the steps of the virus life cycle could, in principle, be disrupted, leading to the destruction of the virus. Disruption of nucleic acid synthesis is perhaps the most common mode of viral inactivation. For example, nucleoside analogs such as AZT and DDI lack the 3′-hydroxyl group, and function as chain terminators. That is, once AZT or DDI are incorporated into the DNA, they prevent the completion of the DNA molecule.[104] Both compounds exhibit anti-HIV activity in vivo; however, the eventual emergence of drug-resistant viral variants likely contributes to the fact that these treatments may delay, but do not completely block, the progression to clinical disease in HIV-infected persons.

Other stages in the virus replication cycle are also amenable to antiviral therepies. Amantidine, an antiviral agent which is clinically useful against the

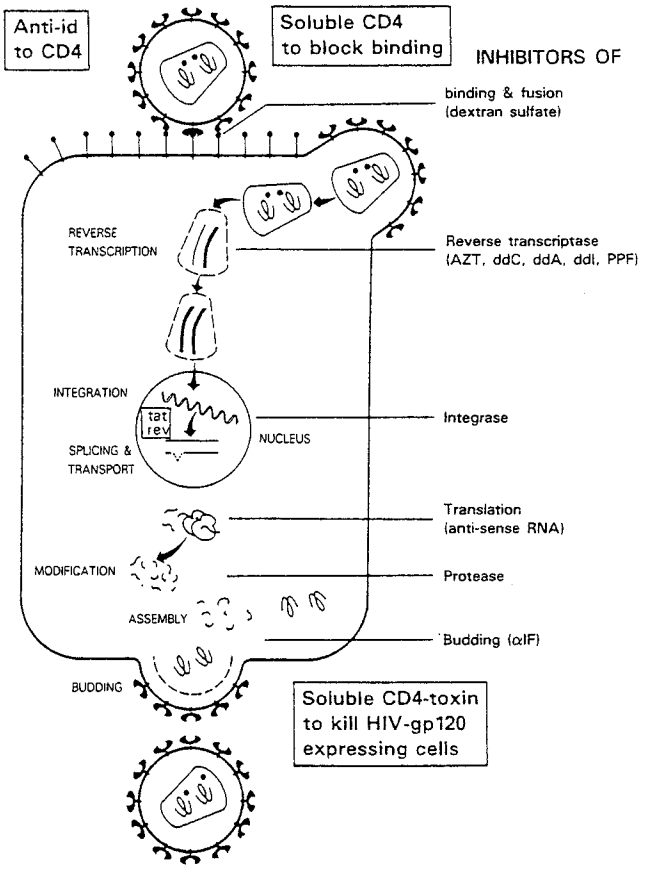
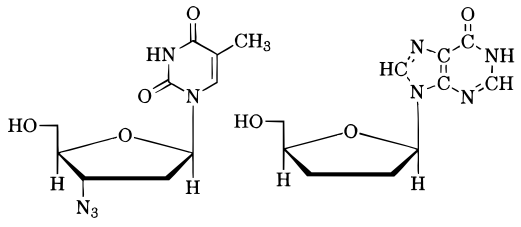
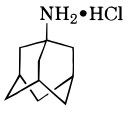


Figure 1. Strategies for blocking HIV replication. This scheme depicts the simple life cycle of HIV replication. Strategies that interrupt retrovirus replication in general, and those for HIV in particular (boxed), are outlined. AZT, ddC, ddA, and ddI are deoxynucleotide analogs that cause chain termination, PPF is phosphonoformate, a reverse transcriptase inhibitor, and αIF is α-interferon. The figure is taken from *Field's Virology*, 2nd ed.; Fields, B. N., Knipe, D. M., Eds.; Raven Press, Ltd.: New York, 1990; Chapter 53, p 1539, and is used with permission.



Azidothymidine

Dideoxyinosine (DDI)



Amantadine

influenza A virus, blocks the uncoating of the influenza A virus by acting as an ion channel blocker. This prevents the establishment of a pH gradient across the viral membrane which normally acts to trigger the disruption of the capsid protein.[1] Inhibition of late stages of HIV replication is being pursued though development of protease inhibitors. HIV-1 protease is a virus-encoded aspartic proteinase which is required for maturation of infectious HIV-1.[98] A number of small peptide molecules which resemble the enzyme substrate have been developed which compete with HIV-1 protease and are in clinical use.[2−4]

A relatively new approach to antiviral therapy is the application of somatic gene therapy. Several strategies have been proposed to target antiviral genes to cells that support replication of HIV-1.[5] The exogenous gene product may interact directly with HIV-1 to inhibit specific stages of virus replication, or may result in destruction of virus-infected cells. In both cases, the overall goal is to prevent further spread of infectious virus.

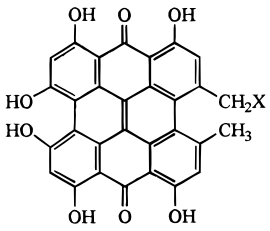
Strategies which interfere with HIV-1 replication include expression of decoy RNAs,[6−9] antisense sequences,[10−13] ribozyme RNAs,[9,14−17] and production of transdominant mutant proteins.[18−25] In many cases, these therapies target the viral regulatory proteins Tat and Rev. These proteins regulate HIV-1 gene expression at the transcriptional (Tat) or post-transcriptional (Rev) level and are required for production of infectious virus. Both Tat and Rev are RNA-binding proteins, and current gene therapy approaches are directed toward preventing, or inhibiting, the specific RNA−protein interaction. A second gene therapy approach involves elimination of virus-infected cells. Direct killing of HIV-1-infected cells has been achieved through expression of cytocidal genes, such as diptheria toxin,[26] or through expression of genes which render the cells susceptible to drug treatment. For example, cells expressing the herpes simplex virus thymidine kinase gene are susceptible to ganciclovir, whereas normal cells are not.[27,28] The use of these therapy strategies requires that gene delivery methods selectively target virus-infected cells and/or are specifically expressed in virus-infected cells. Restricting expression of a susceptibilty gene to HIV-1-infected cells can be achieved by including HIV-1 sequences which interact with viral regulatory proteins Tat and Rev such that the delivered gene is expressed in HIV-1-infected cells, but not in normal cells. For example, Tat increases HIV-1 transcription by binding to a highly structured RNA sequence, termed TAR, present at the 5′-end of all viral mRNAs. The inclusion of TAR sequences in the promoter controlling the susceptibility gene results in increased levels of the toxic protein in HIV-1-infected cells as compared to noninfected cells. When combined with gene delivery systems that target genes to HIV-1 susceptible cells,[29,30] such strategies would theoretically reduce background toxicity.

The high rate of virus replication and mutation often results in the development of drug-resistant mutations and limits the long-term usefulness of many antiviral drugs. Antiviral drugs which have more general toxic effects are less susceptible to virus mutation and may be amenable to gene therapy approaches that target virus-infected cells rather than virus-specific proteins. We have therefore focused our attention on the development of a therapy that would theoretically reduce the appearance of drug-resistant mutants. Our group has characterized the antiviral activity of hypericin using equine infectious anemia virus (EIAV) as a retroviral model of HIV.[31−38] EIAV is a well-suited model for for studies of antiviral agents effective against HIV since

it is an enveloped lentivirus that is structurally, genetically, and antigenically related to HIV.[39−41] Our studies have demonstrated that hypericin, in the presence of ambient room light, reduces the infectivity of cell-free stocks of EIAV by 99.99%.[31] Subsequently, we reported that light was required for the virucidal activity of hypericin,[32] a finding later confirmed by others using HIV-1.[42,43] More recently, we have devised a strategy to place in the proximity of hypericin a chemiluminescent light source so that photodynamic therapy can be extended to all regions of the body.[33] Examination of the structural and chemical basis for light-activated virucidal activity has resulted in new concepts of how these compounds may inactivate the virus.[34−36] These studies represent the fruit of a successful interaction of synthetic chemistry with molecular virology and physical chemistry and illustrate how such interactions may lead to novel antiviral therapies.

Antiviral Activity of Hypericin

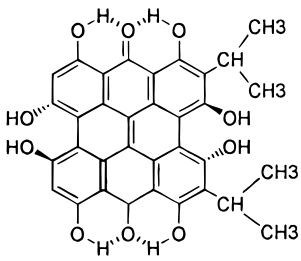
Hypericin (**1**) and its hydroxylated analog, pseudohypericin (**2**), are naturally occurring quinones which



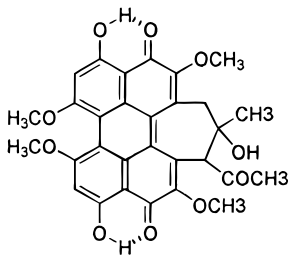
1: X = H
2: X = OH

exhibit potent in vitro inhibitory activity against several enveloped viruses, including retroviruses, herpes viruses, and human immunodeficiency virus (HIV).[31,32,42−47] Hypericin is the pigment in the plant St. John's wort and has a long history of use as a folk medicine for treating ailments as wide ranging as bed wetting, headache, rheumatism, and depression.[48] Extracts from St. John's wort can be purchased in health food stores and have been taken orally by individuals infected with HIV.[49,50] Hypericin is also currently being used in clinical trials for HIV.[49−54] Light-skinned subjects, both human and animals, can develop erythema (irritation and redness of the skin), whereas dark-skinned individuals are much less susceptible to these effects. Studies of photosensitization in animals have shown that the extent of phototoxicity varies with the dosage of the photosensitizer and with the subsequent exposure to light.[96] In AIDS clinical trials, early reports found classical symptoms of photosensitization with relatively high doses of hypericin (0.5 mg/kg).[105] However, as recently as 1993 Steinbeck-Klose and Wernet reported the long term (40 month) successful treatment of HIV-infected persons with no reported side effects.[52] In studies of hypericin as an antidepressant, therapeutic doses (250 μg three times per day) were achieved with no significant toxic side effects.[106] In vivo application of hypericin for treatment of HIV-1 infections would be significantly increased if therapeutic doses were below levels associated with phototoxicity. This may be possible if the mechanism of antiviral activity of hypericin were better understood, thereby allowing the design of compounds with increased virucidal activity and, subsequently, lowered therapeutic dose. Alternately, it may be possible to design novel, light-independent compounds which exert a similar mechanism of antiviral activity. Over the last several years, our group has studied the photophysical properties of hypericin and related compounds in order to better understand the virucidal properties of hypericin.

In 1991, we reported that light was required for the antiviral activity of hypericin.[32] Interestingly, the fluorescent light from the laboratory lighting was sufficient to allow observation of in vitro activity. More recent studies demonstrated the tumoricidal activity of hypericin and found it to be dependent on the presence of light and oxygen.[56] Therefore, hypericin is similar to other photosensitizers that, when activated by light, exert potential therapeutic effects against tumors and some viruses. Related compounds such as stentorin, hypocrellin A, and many



Stentorin



Hypocrellin A

porphyrins and phthalocyanines are members of this class.[57] There is a rapidly-expanding literature on the use of photodynamic compounds to inactivate tumor cells and virus-infected cells (photodynamic therapy) that has been collated in excellent reviews by Song and Diwu.[58,99]

In recent years the therapeutic use of photosensitizers has emerged as a promising tool in cancer chemotherapy.[59] The mechanism of cytotoxicity most probably involves the generation of singlet oxygen by the light-excited photosensitizer.[60,61] Hypericin produces singlet oxygen very efficiently (with a quantum yield of 0.73[62]), and singlet oxygen is most probably involved in hypericin-mediated killing of tumor cells in vitro.[56] Other studies have suggested that the antiviral activity of hypericin is due to the production of singlet oxygen.[42,43,47,63] However, the excited-state reactivity of hypericin extends well beyond the photosensitization of oxygen to form singlet oxygen. In fact, recent studies suggest that antiviral activity may be due to complex mechanisms involving the superoxide anion and hypericinium ion. Redepenning and Tao[64] have measured the formal potential of hypericin in DMSO by cyclic voltametry and concluded that, in its excited state, hypericin is both a good oxidizing and reducing agent. Diwu and Lown observed both singlet oxygen and superoxide radical upon illumination of hypericin with 580-nm light under aerobic conditions.[65] They also indirectly observed the formation of a semiquinone radical species in the absence of oxygen. Mazur and coworkers have obtained similar results.[66,67]

We have demonstrated that hypericin undergoes an excited-state intramolecular proton transfer and that it is an excited-state proton source. We have hypothesized that photogenerated protons may be important in the antiviral activity of hypericin.[35−38] Our finding that hypericin retains antiviral activity under hypoxic conditions[34] underscores the potential importance of these excited state intermediates in the virucidal activity of hypericin.

The mechanisms by which photoactivated hypericin targets and inactivates viruses are not clear. Because of the potential importance of hypericin as a therapeutic agent for treatment of HIV-1 infection, most information available on the antiviral activity of hypericin derives from studies of HIV[42,43,47,68] and related retroviruses.[31,32,47] These studies have demonstrated that hypericin is able to inactivate cell-free virus and to inhibit virus production from infected cells. Meruelo and co-workers[43,69] have observed that, in the presence of light, hypericin induces significant changes in the HIV-1 capsid protein, p24, and the p24-containing *gag* precursor, p55, as indicated by Western blot analysis. They have also observed that recombinant p24 in the presence of light forms an immunoreactive material of a molecular weight of 48 kDa. They have consequently suggested that cross-linking and other alterations of p24 occur and that such alterations may inhibit the reverse transcriptase activity. In other studies, inhibition of gp120 binding was observed under conditions of more intense illumination.[43] Together, these results indicate that observed differences in the biological effects of photoactivated hypericin may depend on the level of irradiance and concentration of photosensitizer.

Molecular Flashlight

In vivo applications of hypericin for treatment of HIV-1-infected individuals is hampered by the dependence on light for optimal antiviral activity. Some therapeutic benefit may be achieved due to the fact that the longer wavelengths of light at which hypericin absorbs (Figures 2 and 6) are fairly good at penetrating the body. Therefore, systemic irradiation following hypericin treatment may result in a reduction in viremia and destruction of virus-infected peripheral blood leukocytes. Hypericin has been tested in phase I clinical trials, and there is some evidence that long term treatment may slow the progression of clinical disease. However, this therapeutic approach may be of limited effectiveness in elimination of HIV-1 in lymph nodes. Virus replication and persistence in lymphoid tissue play a major role in HIV pathogenesis.[70−72] Our approach has been to develop a strategy to place in the proximity of hypericin a chemiluminescent light source so that photodynamic therapy can be extended to all regions of the body. Moreover, it occurred to us that the requirement for light activation might enable us to develop a drug which would target only virus-infected cells.

An expedient choice for the light source is luciferin (Figure 2). The reaction of luciferin with the enzyme luciferase and molecular oxygen produces light in the 520−680-nm region with a quantum efficiency of about unity.[73] Hypericin absorbs light strongly in this range, suggesting that some combination of energy transfer and reabsorbtion between the product of the chemiluminescent reaction (Figure 2) and hypericin may be sufficient to effect significant antiviral activity. In vitro studies indicated that, at high concentrations of hypericin, there is approximately a 10-fold reduction of viral infectivity under conditions where the sole source of excitation was the chemiluminescent luciferin/luciferase system (Figure 3). The chemiluminescent light-generating system was not, however, as effective in activating hypericin as illumination from a continuous source. Further experiments were done to determine if increased antiviral activity could be achieved by an increase

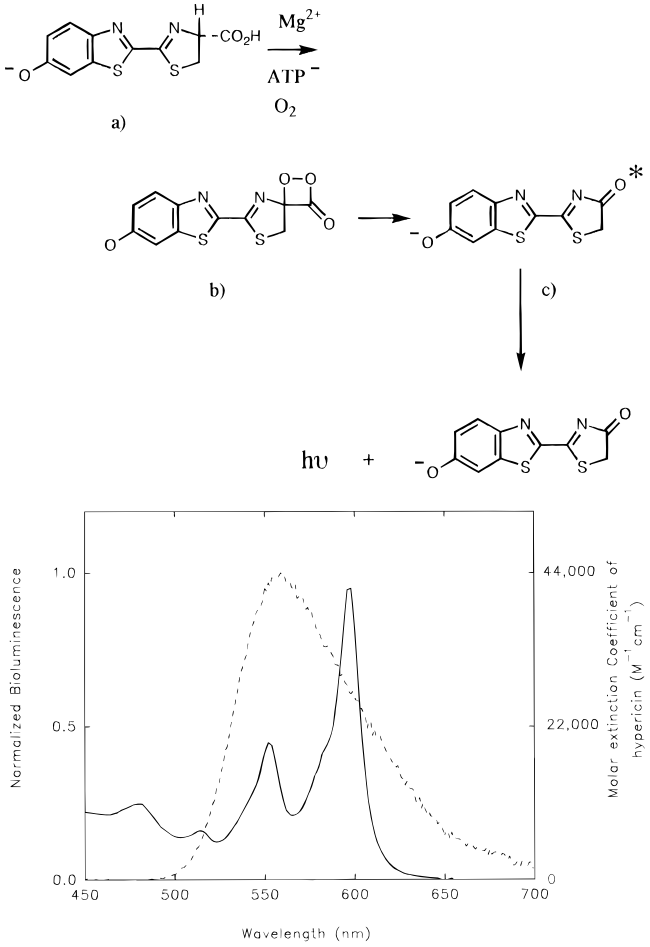


Figure 2. Above are displayed crucial intermediates in the production of firefly chemiluminescence.[33] Luciferin (a) is catalyzed by the enzyme luciferase in the presence of ATP, Mg^{2+}, and O_2 to form the high-energy four-member peroxide or dioxetanone intermediate (b). This intermediate subsequently decarboxylates to form the chemilumnescent species oxyluciferin (c). Below is presented the spectral overlap between the visible portion of the absorption spectrum of hypericin and the chemiluminescence from the luciferease-catalyzed oxidation of luciferin. The reaction is carried out at 25 °C in glycylglycine buffer containing 2.67×10^{-7} M luciferase; 1.18×10^{-6} M luciferin, and 5×10^{-5} M ATP. The efficiency of the nonradiative energy transfer in a Förster energy transfer mechanism is given by R_o, the critical distance, which in this case is 100 Å. Both radiative as well as nonradiative energy transfer are likely to occur in this system. If we identified a way to ensure that the interaction of luciferin with luciferase in the presence of hypericin took place only in virus-infected cells, then we would have a selective and effective antiviral therapy.

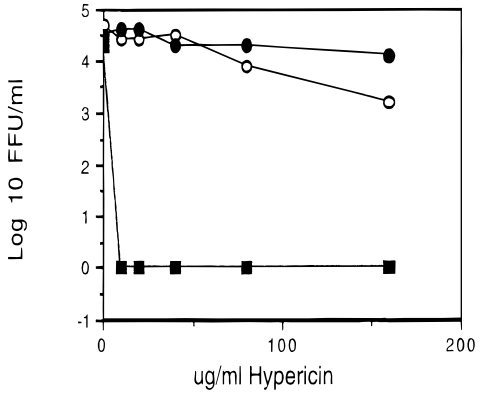


Figure 3. Effect of chemiluminescence on the antivral activity of hypericin. EIAV was incubated in the dark at room temperature in the presence of 0.8 μM luciferase, 10 μM luciferin, 2 mM ATP, and increasing amounts of hypericin (○). Control samples include those containing virus and hypericin only (●) and parallel samples exposed to ambient room light (■). Infectious virus was titrated by using a focal immunoassay,[31,33,34,103] and the results are reported as focus forming units (FFU) per milliliter of reaction mixture.

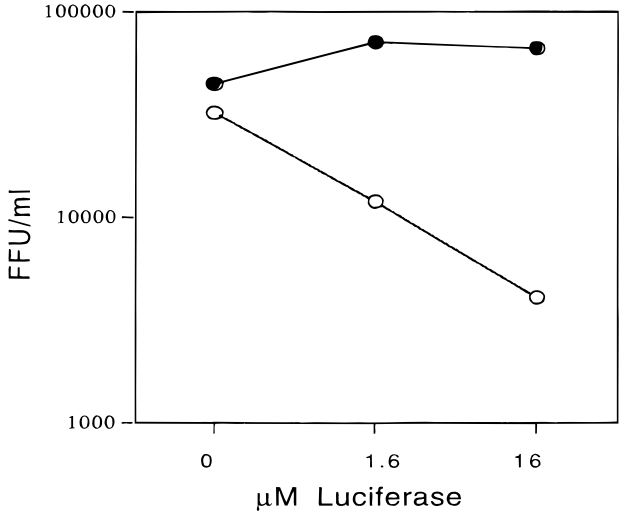


Figure 4. Effect of luciferase concentration on antiviral activity of hypericin after chemiluminescence. EIAV was incubated with 0 (●) or 10 (○) μg of hypericin/mL in the presence of 5 mM luciferin, 2 mM ATP, and increasing amounts of luciferase. Reaction mixtures were incubated in the dark for 30 min at room temperature, and results are reported as FFU/mL of reaction mixture.

in the amount of light initially available for hypericin activation. A linear decrease in viral infectivity was observed when the concentration of luciferase was varied in the presence of a constant concentration of hypericin (Figure 4).

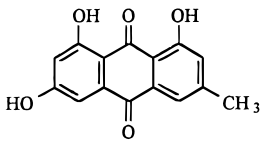
One reason that the chemiluminescent system is not as effective in activating the antiviral activity of hypericin as is a continuous source of illumination is that there is most likely a suboptimal distance and orientation between the donor and acceptor. Nevertheless, energy transfer between luciferin/luciferase and hypericin is possible even when the donor and the acceptor are not constrained to be at a fixed distance or orientation with respect to each other or when there is no covalent attachment between these two reactants. Most importantly, the amount of light transferred to hypericin under these conditions is sufficient to produce significant antiviral activity.

The finding that the antiviral activity of hypericin can be activated by chemiluminescent reactions may have important implications for the development of novel methods for treatment of viral infections such as HIV-1. In vivo generation of luciferase could be accomplished using gene therapy approaches that employ luciferase as a susceptibility gene. Moreover, expression of the luciferase gene could be regulated if placed under the control of a promoter containing HIV TAR sequences, limiting photoactivation of hypericin to virus-infected cells. This would result in a "molecular flashlight" in which light is turned on or off, depending on the presence of the HIV-1 transacting viral protein Tat.

Synthesis of Hypericin, Its Analogs, and Components of the Molecular Flashlight

With a technique for activating hypericin now available, we focused our efforts on the problem of drug targeting. Any in vivo application of chemiluminescent activation of hypericin would, of course, require a delivery system that ensures a high local concentration of hypericin and luciferin/luciferase. In order to avoid the problems inherent in requiring three separate compounds to come together in the virus-infected cell, we decided to covalently connect hypericin and luciferin. This tack required an efficient synthesis of both hypericin and luciferin. Although both compounds are commercially available, hypericin is available in limited quantities and is quite expensive ($400/10 mg). Luciferin is also rather expensive ($86/10 mg). Apparently, much of the luciferin supply comes directly from fireflies.

Existing syntheses of hypericin begin with emodin (**3**). Steglich and Rodewald independently reported the synthesis of hypericin in one step, albeit in approximately 1% yield, by the treatment of emodin with dilute aqueous base at 100 °C for ten days.[75] Apparently, the coupling of two emodin molecules affords dimer **4** as the predominant initial product.



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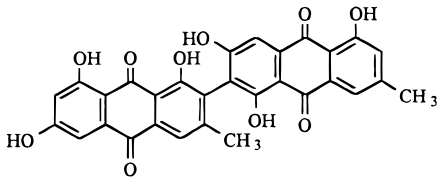

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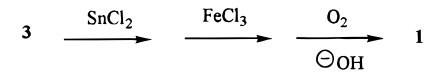
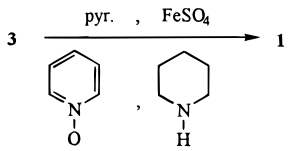
The initial dimer that could lead to hypericin is a minor product. Banks, Cameron, and Raverty also reported a synthesis of hypericin from emodin.[76] Their synthesis is depicted below. This synthesis



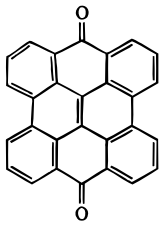
involves the reduction of emodin to an anthrone, coupling the anthrone with ferric chloride to a

bianthrone and oxidation of the bianthrone in ammonia with oxygen.

Although the yield on a millimole scale is reported to be good, this reaction appears to be difficult to scale up to generate gram quantities of hypericin. Another synthesis of hypericin from emodin has been reported in a patent.[100] This reaction is depicted below.



In designing hypericin conjugates for antiviral therapy, it is important to know which functional groups on hypericin are essential for potent antiviral activity. To address this question, we synthesized analogs **5**−**10**.



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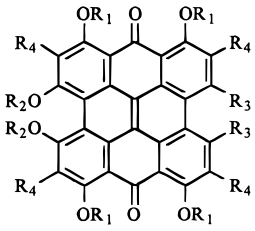
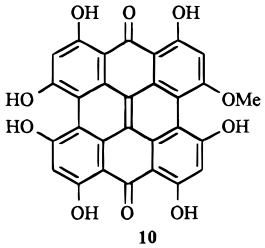


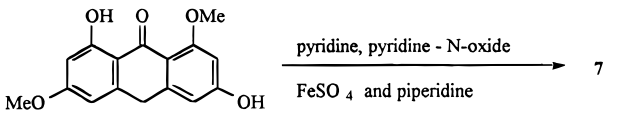


10

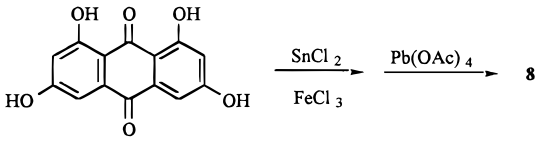
6: $R_1=R_2$=Me, R_3=OMe, R_4=H
7: R_1=H, R_2=Me, R_3=OH, R_4=H
8: $R_1=R_2$=H, R_3=OH, R_4=H
9: $R_1=R_2$=H, R_3=OH, R_4=Me

We had previously reported that hydroxylated anthraquinones, hydroxylated perylenequinones, and polycyclic quinones which resembled hypericin did not exhibit significant antiviral activity.[31] Analogs **5**[37,77] to **10** assess the relative importance of hydroxyl groups on the phenanthro-perylene ring. Analog **5** is a known compound that was synthesized from anthrone in two steps.[77] Analog **6** was prepared from the anthrone of 1,3,6,8-tetramethoxyanthraquinone. On the basis of ultrafast spectroscopic studies of hypericin and related hydroxyquinones, we have proposed that the hydroxyl groups peri to the quinone carbonyl groups contribute to the antiviral activity in the presence of oxygen and are essential to the antiviral activity in the absence of oxygen.[34−38] The ultrafast spectroscopic measurements of analog **6** will test this hypothesis.

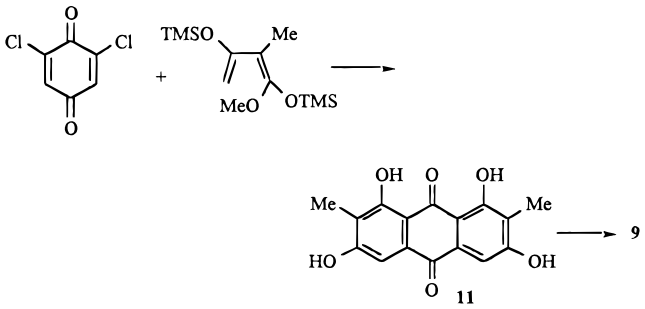
Analog **7** was synthesized as shown below. Analog **10** was produced in low yield as a byproduct of the synthesis of **7**.



Analog **8** was readily prepared from 1,3,6,8-tetrahydroxyanthraquinone by coupling of the corresponding anthrone followed by oxidative cyclization. We have found that lead tetraacetate is a superior oxidant in this case.



Anthrone analog **9** was efficicently prepared from 1,3,6,8-tetrahydroxy-2,7-dimethylanthraquinone (**11**),



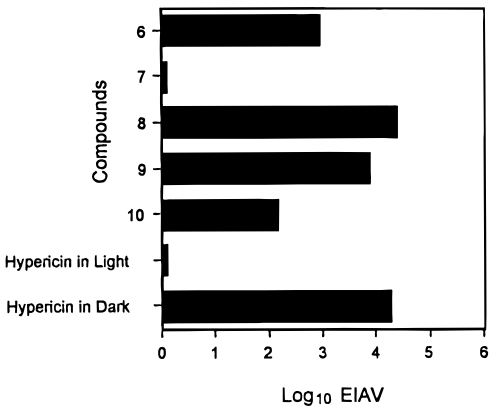
which in turn was synthesized from 2,6-dichlorobenzoquinone by a double Diels−Alder reaction. Since anthraquinone **11** has only one site for radical coupling, we tried to make **9** using the conditions developed by Steglich. Unfortunately, after **11** was treated with base for 7 days at 100 °C, only starting material was recovered. However, subjecting **11** to reduction ($SnCl_2$), coupling ($FeCl_3$), and oxidation ($PbOAc_4$) furnished analog **9** in 68% yield.

Analogs **5**−**10** were tested for anti-retroviral activity in vitro using EIAV.[32] Antiviral activity is determined by the amount of EIAV present, as measured by the number of focus-forming units per milliliter (FFU/mL)[32] (Table 1). Analogs **5**,[31] **8**,[107] and **9** did not exhibit any antiviral activity, and analogs **6** and **10** possess antiviral activity that is considerably reduced with respect to hypericin.

On the basis of the discussion of excited-state processes below, it is tempting to attribute the absence of antiviral activity in **5** and **6** to the absence of labile protons. It is possible, however, that **5** and **6** produce much less singlet oxygen than does hypericin, and we have not ruled this out. The UV−vis spectrum of **9** resembles that of deprotonated hypericin. Again, it is tempting to attribute its reduced antiviral activity to the absence of labile protons.

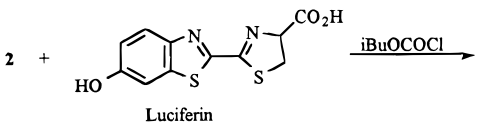
Structurally, the differences between **8** and **10** are very slight (a hydrogen atom substituted by a methyl group). An explanation for the reduced antiviral activity in **10** and the absence of antiviral activity of **8** is that because **10** and **8** contain, respectively, four and three more hydroxyl groups than does hypericin, these molecules are more soluble in water than hypericin. Consequently, **10** and **8** are not as efficiently driven into the viral membrane. It is well-known that hypericin is exceedingly insoluble in water except at very acidic or basic pH (see below).

Table 1. Antiviral Activity of Hypericin Analogs

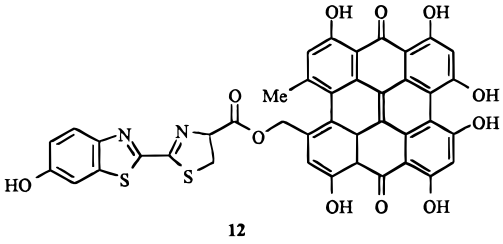


The antiviral activity of hypericin and its analogs is likely the net result of an interplay between the redox potential and the molecule's ability to partition between the cell membrane and water. The construction of conjugates that retain the potent antiviral activity of hypericin seems limited to functionalization of the hydroxyl groups not involved in hydrogen bonding to the carbonyl group.

The analog study also provided valuable insight into the synthetic limitations involved in the preparation of a suitable tethered molecule. In particular, the high antiviral activity of **7** (comparable to that of hypericin) appears to dictate the choice of sites for connection of luciferin to hypericin. In light of the above information, we prepared the tethered molecule **12**. Since esters of luciferin do not react with



Luciferin



12

luciferase, this tethered molecule must be cleaved in order to afford luciferin. Cellular enzymes that cleave esters are readily available in host cells. Therefore, we reasoned that, once compound **12** passed through the cell membrane, it would soon be cleaved into the two components. Concerns relating to subcellular localization of the luciferase and hypericin and luciferin could readily be evaluated. An important result is that the synthesis of **12** allows us to test experimentally the ability of the molecular flashlight to inhibit virus replication in infected cells.

Using Molecular Biology To Target the Antiviral Activity of Hypericin

As a model system for development of inducible chemiluminescence, we constructed a plasmid containing the promoter region of EIAV inserted upstream of the gene for luciferase (pMA-1LTRLuc). This luciferase plasmid was cotransfected with a neomycin resistance plasmid (pSV2Neo) into Cf2th cells (ATCC CRL1430) using calcium phosphate coprecipitation. Cells were selected for neomycin resistance, and the level of luciferase activity was quantitated as a function of light generated following the addition of 400 μM luciferin to 10-fold serial dilutions of cell lysate.

Table 2. Fold-Transactivation of Luciferase Expression by EIAV in Stable Cell Lines Transfected with pMA-1LTRLuc

	nM luciferase		
cell clone	uninfected	MA-1 infected	fold-increase
13-2	0.13	1.9	15
13-4	0.008	0.95	118
14-1	0.14	1.0	7
14-2	0.19	1.5	7
14-3	0.02	1.1	55
17-1	0.007	0.65	9
17-4	0.017	1.7	100
18-1	0.015	0.66	44
18-2	0.023	2.1	91
18-3	0.006	0.74	123
18-4	0.002	2.0	1000
19-1	0.016	1.5	94

Positive cells were subcultured, and parallel cultures were infected with the MA-1 isolate of EIAV. Chemiluminescence assays were done to determine if increased levels of luciferase expression occurred in virus-infected cells. Results (Table 2) indicated that luciferase expression increased 10−1000 fold in virus-infected cells, as compared to noninfected cells. The matched cell lines 18-4 and 18-4/MA-1 provided a useful model to test the tethered compound **12**. Specifically, we wanted to know if chemiluminescent activation of hypericin would reduce production of a lentivirus in cells expressing luciferase.

Cells infected with EIAV (Cf2th/EIAV) and EIAV-infected cells expressing the luciferase gene (18-4/EIAV) were treated with the tethered compound **12** and incubated at 37 °C for 1 h. After extensive washing, culture supernatant was sampled from each well and virus production was quantitated. Results indicated that production of infectious virus was reduced at least 10-fold in 18-4/EIAV cells treated with **12** as compared to untreated cells; no reduction in infectious virus was observed in Cf2th/EIAV. These results suggest that treatment of lentivirus-infected cells expressing the luciferase gene with a hypericin-luciferin tether can generate sufficient chemiluminescence to activate the antiviral activity of hypericin. Current efforts are focused on optimizing this system for sustained, high levels of antiviral activity in persistently infected cells.

Excited-State Processes in Hypericin and Their Role in Light-Induced Antiviral Activity

Elucidating the reactivity of excited-state hypericin and the subsequent reactivity of molecules that it encounters is essential for understanding the light-induced mechanism of antiviral activity[32,42,43,47,62,64−67,69,78] as well as for developing optimal antiviral agents or alternative antiviral strategies.

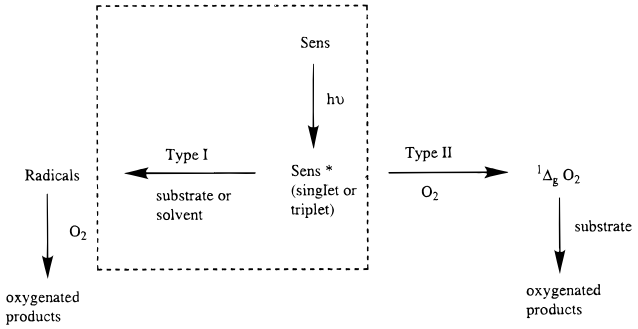


Figure 5. Type I and type II photosensitization processes. Type I and II processes are respectively distinguished by whether the photosensitizer encounters the substrate or O_2 first.

The mechanism of photosensitization by a given molecule in its excited state can be classified into two types of processes[56,79] (Figure 5). In type I processes, the excited-state photosensitizer interacts first with the substrate, which may go on to react with another reagent, which is usually oxygen. In type II processes, the excited-state photosensitizer interacts first with oxygen, thus producing singlet oxygen, which subsequently goes on to react with the substrate. We[36−38] have questioned the relative importance of singlet oxygen in the toxicity of hypericin toward HIV and related viruses. For example, hypericin is closely related,[80] both structurally and spectrally, to the photoreceptor of the protozoan ciliates *Stentor coerulus*[80,81] and *Blepharisma japonicum*.[82] This photoreceptor confers upon the organism its biologically necessary photophobic and phototactic responses. Under conditions of ambient light the stentorin chromophore and hypericin are nontoxic to the organism. On the other hand, the singlet oxygen produced from these chromophores is toxic to *S. coerulus* under high light flux (∼5000 W/m^2).[83] It is an open question, therefore, whether the limited exposure to room light in our experiments[32] and those of other workers[42,43,47,69] is toxic to EIAV, HIV, and other enveloped viruses because of photosensitized generation of singlet oxygen by hypericin or because of the presence of additional nonradiative decay processes of the excited states of hypericin.

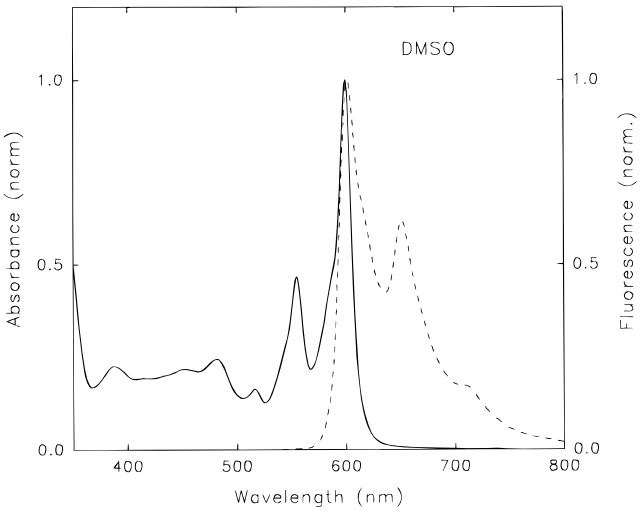


Figure 6. Normalized fluorescence spectrum (dashed line) and absorption spectrum (solid line) of hypericin in DMSO. The spectra of hypericin are very similar in all solvents in which it is soluble with the exception of shifts in absorbance and emission maxima. (Hypericin in water at pH values between 3 and 11 is barely, if at all, soluble and is nonfluorescent.) The steady-state emission spectrum bears a "mirror symmetry" relationship to the visible portion of the abosrption spectrum. We attribute this part of the absorption spectrum to the presence of ground-state tautomer.

Steady-State and Time-Resolved Spectroscopy of Hypericin and Its Analogs: Excited-State Proton Transfer

We have provided the first detailed investigation that uses both ∼1-ps time resolution and a white-light continuum to examine and to unravel the excited-state primary photoprocesses of hypericin and have suggested that the excited-state transients we observe, coupled with data from model compounds, can be interpreted in terms of tautomerization[36−38] based on the following observations.

(1) Model compounds demonstrate that a protonated carbonyl group is required in order to obtain hypericin-like absorption and emission spectra.

Figure 6 gives the steady-state absorption and fluorescence spectra of hypericin in DMSO. In contrast to hypericin, its deshydroxy analog, mesonaphthobianthrone (**5**), is nonfluorescent in the aprotic solvents DMSO (Figure 7a) and CH_3CN. When, however, **5** is dissolved in a protic solvent such as methanol (in which it is only sparingly soluble), a fluorescence band appears with a maximum at 467 nm (Figure 7b). Finally, dissolving **5** in a strong acid

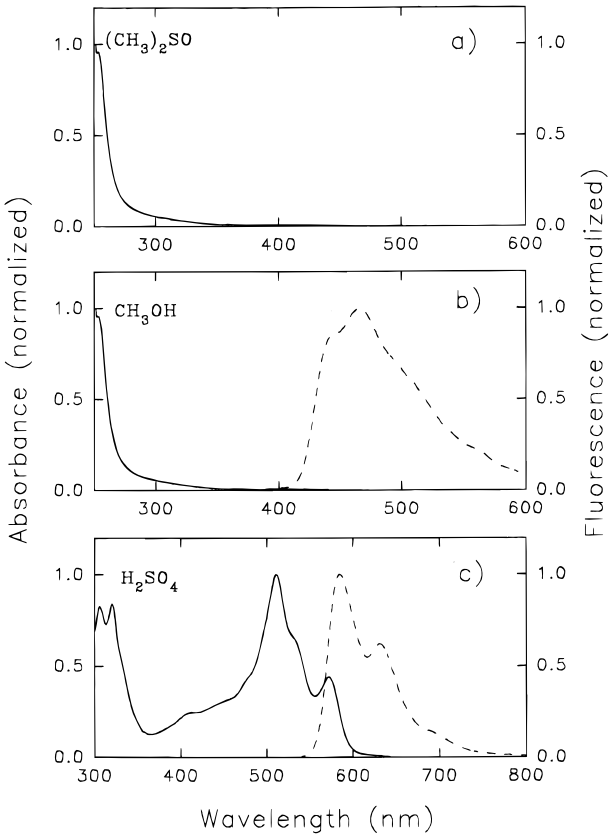


Figure 7. Normalized fluorescence spectra (dashed line) and absorption spectra (solid line) of the hypericin analog lacking hydroxyl groups, mesonaphthobianthrone. The solvents used are DMSO (a), methanol (b), and sulfuric acid (c).

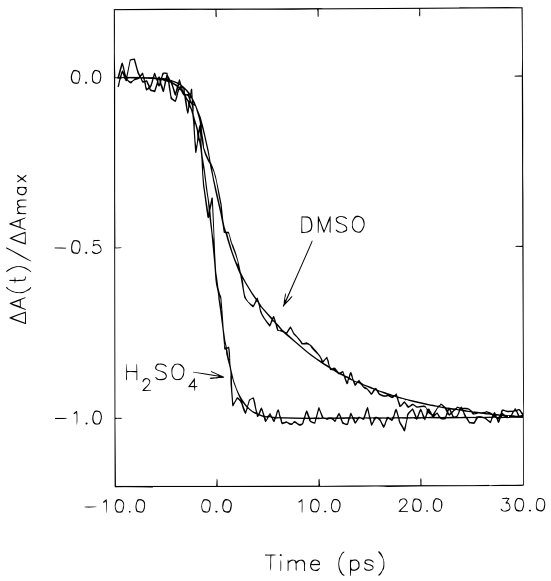


Figure 8. Comparison of the rise time for the formation of excited-state transients. This rise time is finite for hypericin in an aprotic solvent where a portion of the ground-state population is not tautomerized (DMSO). But in a solvent where the entire ground-state population is protonated (H_2SO_4), the rise time is instantaneous. The fits to the data are as follows. DMSO: $\lambda_{probe} = 610$ nm, $\Delta A(t) = 0.23 \exp(-t/9.6) - 0.41$. H_2SO_4,$\lambda_{probe} = 630$ nm, the transient appears within the time resolution afforded by the system, and the excited state formed is long-lived on the time scale of the measurement.

such as sulfuric or triflic acid generates a fluorescence spectrum that has nearly the same shape as that of hypericin in DMSO and that is blue-shifted from the hypericin spectrum by about 14 nm. The emission maximum of **5** is 584 nm. These results demonstrate the importance of a protonated carbonyl group for producing a fluorescent hypericin-like molecule.

(2) Time-resolved absorption (stimulated emission) spectra and kinetics indicate that the hypericin emission spectrum grows in on a 6−12-ps time scale. On the basis of the model compounds, the rise time for the appearance of the hypericin emission is taken as evidence for an excited-state proton transfer. Figure 8 is very instructive in this regard. In sulfuric acid, where all the carbonyl groups are expected to be protonated, the excited-state transient appears instantaneously. On the other hand, in a solvent such as DMSO, the transient appears with a finite rise time. The excited-state absorbance transients are of particular interest due to the earlier observations and suggestions of Song and co-workers[84,85] that the excited states of hypericin-like chromophores produce protons upon photoexcitation.

Potential Antiviral Role of Excited-State Processes: O_2 Is Not Required for Photoinduced Antiviral Activity of Hypericin

The influenza A virus provides an interesting example that demonstrates the importance of pH in viral infectivity. The influenza A virus consists of an envelope comprised of a lipid bilayer membrane and associated membrane proteins. This envelope surrounds the genomic RNA, which is protected by capsid proteins. The envelope of influenza virus A contains two proteins the function of which in the virus life cycle is pH dependent:[93] hemagglutinin (HA) and M_2. The influenza virus binds to the cell surface by means of HA, and it enters the cell through receptor-mediated endocytosis. Within the endosome, the low pH (∼5−6) causes a conformational change in HA, which results in the fusion of the viral membrane with the membrane of endosomal vesicles inside the cell. M2 spans the viral membrane and functions as an ion channel to modulate the pH within the virion and facilitate the uncoating of the genomic RNA. Thus, changes in ion concentration are required for influenza virus entry and uncoating.

To explore the possible role of excited-state proton transfer in anti-retroviral activity, we have compared the antiviral activity of hypericin under aerobic and hypoxic conditions.[34] Hypericin is toxic in the presence and the absence of oxygen (although the data suggest that hypericin in a hypoxic environment does not inactivate EIAV as effectively as in an oxygenated environment.) These results demonstrate that direct interaction of hypericin itself with the virus (type I mechanism) can be important for the remarkable antiviral properties of hypericin. The relative importance of type I and type II mechanisms of hypericin under normal biological conditions is as yet unclear.

It is useful to consider our experiments in the context of other reports on the biological activity of hypericin. For example, the stentorin chromophore is toxic to *S. coerulus*[83] under high, but not ambient or low, light flux. Also, Meruelo and co-workers[42,43] found that, under ambient lighting conditions, hypericin did not inhibit the binding of gp120 to CD4 cells and that it did not inhibit the formation of syncytia (large, abnormal multinucleated cells formed by the fusion of infected cells with uninfected CD4 cells).[42,43] On the other hand, inhibition of gp120 binding was observed under conditions of more intense illumination: i.e., when samples were placed ∼10 cm away from a fluorescent light source for 30 min.[43]

Therefore, the relative importance of type I and II mechanisms in virus inactivation may depend on the intensity of light. Under high irradiance, the formation of singlet oxygen by a type II mechanism would be predominant, whereas under low irradiance the antiviral activity would be primarily due to a type I reaction. Such reasoning may explain the differences among investigators in characterizing the antiviral mechanism of hypericin cited above. At high irradiance, there is both inhibition of gp120 binding and inhibition of cell fusion,[42,43] events that depend on interactions between virus and cell membranes. Interestingly, the effects are not reported to occur at low irradiance,[42] where inhibition is associated with alterations in the viral capsid protein. This may indicate that type II reactions target the viral membrane, whereas type I reactions may attack other stages in the life cycle of the virus. This may also explain the absolute dependence on oxygen for the anti-tumor effect of hypericin.[56]

We are performing similar investigations on the perylene quinone analog of hypericin, hypocrellin. Hypocrellin has been observed to have light-induced antiviral activity against HIV.[87] Our measurements

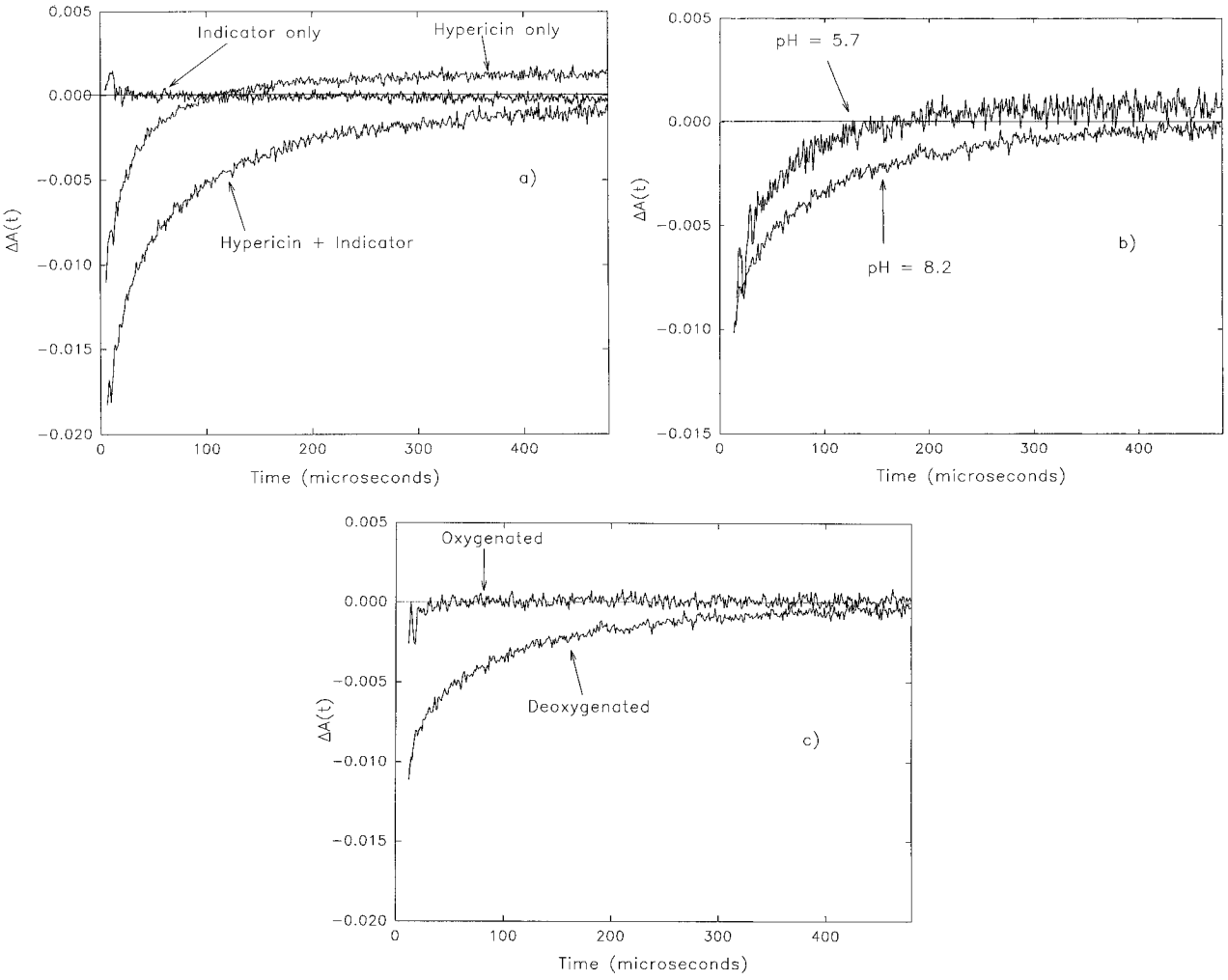


Figure 9. (a) Transient absorption at 400 nm of 3-hexadecanoyl-7-hydroxycoumarin subsequent to excitation of hypericin with a 500 ns pulse at $\lambda_{ex} = 490$ nm. Both the indicator (23.3 mM) and hypericin (21.0 mM) are contained in the bilayer of DPPC vesicles at pH 8.4. The decrease in the anionic form of the indicator owing to excited-state protonation by hypericin monitored by a transient reduction of the induced bleach of the anionic form of the indicator at 400 nm. $\Delta A(t) = -0.012 \exp(-t/32\text{ ms}) - 0.0050 \exp(-t/170\text{ ms})$. The control experiment for hypericin alone in vesicles at pH 8.3 yields a trace that is fit to the form $\Delta A(t) = -0.0062 \exp(-t/15\text{ ms}) - 0.0067 \exp(-t/55\text{ ms}) + 0.0080$. A second control experiment using only the indicator alone in vesicles at pH 8.3 yields the trace about zero. This trace demonstrates that in the absence of hypericin no transient absorption is induced in the indicator at 400 nm subsequent to excitation at 490 nm. (b) Induced bleaching and its recovery at 400 nm for 3-hexadecanoyl-7-hydroxycoumarin and hypericin in DPPC vesicles at acidic and basic pH; $\lambda_{ex} = 490$ nm. (c) Induced bleaching and its recovery at 400 nm for 3-hexadecanoyl-7-hydroxycoumarin and hypericin in DPPC vesicles in oxygenated and deoxygenated solution: $\lambda_{ex} = 490$ nm. The absence of a signal in the oxygenated sample is taken as proof that the triplet state of hypericin is responsible for the protonation event. (Under oxygen levels at which the signal is quenched, hypericin is still fluorescent and the indicator absorption spectrum remains unchanged. Consequently the absence of the signal cannot be a result of quenching the singlet state or of destruction of the indicator.)

indicate the following:[102] (a) Hypocrellin exhibits excited-state transients that are longer than those observed in hypericin but that can also be assigned to intramolecular proton transfer. For example, the rise time for the induced absorption is instantaneous in H_2SO_4, where the carbonyl protons are all expected to be protonated. (b) We have found, however, that unlike hypericin, hypocrellin requires oxygen for antiviral activity. (c) Unlike hypericin, hypocrellin does not display light-induced acidification of the solvent medium in measurements analogous to those performed upon hypericin (see below).

The absence of light-induced acidification in hypocrellin and its absolute dependence upon oxygen for antiviral activity are consistent with and support our previous interpretation of the data for hypericin: namely, that hypericin is an excited-state proton source and that protons may play a crucial role in antiviral activity. (Despite this interpretation, we have not dismissed and are currently investigating the possibility that hypericin also executes relevant excited-state redox chemistry.)

Light-Induced Acidification by Hypericin

Recent results from our laboratory indicate that hypericin does indeed act as an excited-state proton source.[35] Steady-state illumination of a solution containing hypericin effects a pH drop as monitored by the indicator, BCECF (2′,7′-bis(2-carboxyethyl)-5-(and 6-)carboxyfluoroscein). When hypericin and an indicator dye, 3-hexadecanoyl-7-hydroxycoumarin, are both imbedded in vesicles, hypericin is observed to transfer a proton to the indicator within a time commensurate with its triplet lifetime. Proton trans-

fer to the indicator is not observed when the indicator is protonated or when the system is oxygenated. Since hypericin is known to form triplets and to generate singlet oxygen with high efficiency, this latter result is taken to confirm triplet hypericin as a source, but not necessarily the only source, of protons. These studies were undertaken in phospholipid vesicles suspended in an aqueous medium in order to circumvent the insolubility of hypericin in water, to maintain a relatively close proximity of the proton donor and acceptor, and to provide a simplified model of the viral membrane, within which hypericin is thought to partition.

The steady-state absorption and fluorescence spectra of hypericin in DPPC vesicles in water at pH 8.4 resemble those of hypericin in DMSO or alcohols (Figure 2), which indicates that aggregation is not occurring. Figure 9a presents the transient at 400 nm of the 3-hexadecanoyl-7-hydroxycoumarin indicator subsequent to pulsed excitation of hypericin at 490 nm. The transient is a bleach the recovery of which is represented by two time constants: 32 and 170 ms. This signal is interpreted as proton transfer from hypericin to the indicator on a time scale commensurate with that of the lifetime of triplet hypericin. Protonation of the anionic form of the indicator decreases its population and consequently reduces its absorbance at 400 nm. The persistent bleach of the indicator at long times can be attributed to the slow re-establishment of equilibrium between the acidic and basic forms of the indicator.

Another confirmation of the assignment of the trace in Figure 9a to a transfer of a proton from hypericin to the indicator is that if the long-lived component is due to the release of a proton from hypericin, its amplitude should be "diminished" by lowering the pH. This result is in fact observed (Figure 9b). Also, molecular oxygen efficiently quenches triplet hypericin to form singlet oxygen. Consequently, at sufficiently high oxygen levels, the concentration of triplet hypericin should be negligible. Figure 9c demonstrates that when the system of hypericin and the indicator is oxygenated, no bleaching of the indicator is observed, as is expected if the triplet is the proton source.

Summary

In this article, we have discussed the absolute dependence of light for antiviral activity in hypericin and how we are exploiting this dependence, through techniques of synthetic organic chemistry and molecular biology, to produce antiviral agents that are capable of specifically targeting virus-infected cells. We have also discussed the role of light in generating excited states that possess antiviral activity and the mechanisms by which these excited states may exert this activity. Consequently, the tightly knit combination of the techniques of synthetic organic chemistry, molecular biology and virology, and physical chemistry can be used not only to develop specificity in an antiviral agent but also to provide a basis for modifying and improving this antiviral activity.

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