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Anti-human cytomegalovirus activity and toxicity of sulfonated anthraquinones and anthraquinone derivatives

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Abstract

Sulfonated anthraquinones and other anthraquinone derivatives were evaluated for anti-human cytomegalovirus (HCMV) activity, cytotoxicity and genotoxicity. Acid blues 40 and 129, acid black 48, alizarin violet R and reactive blue 2 were the most active compounds having selective indices of greater than 30 and EC_{50} values of 4–30 μM. When tested against a clinical isolate, the 4 compounds were 2- to 5-fold less active. The antiviral activity was distinctly separate from the virucidal activity (> 1000 μM). The compounds were weakly toxic to either log phase or stationary cells in most of the following cytotoxicity assays: neutral red uptake assay, lactic acid dehydrogenase assay, trypan blue exclusion assay and radiolabeled macromolecular precursor uptake assays. Using a genotoxicity assay, the comet assay, only reactive blue 2 and acid black 48 were found to cause DNA strand breakage. This occurred at concentrations of 30 and 170 μM, respectively. These results suggest that these compounds could be a prototype for synthesizing even more effective HCMV-inhibitory anthraquinone derivatives.

Keywords: Anthraquinone; Human cytomegalovirus; Comet assay; Genotoxicity assay; Antiviral agent

1. Introduction

Human cytomegalovirus is an opportunistic pathogen causing significant morbidity and mortality in immunocompromised individuals (Grundy, 1990). Because of this, an

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intensive search for novel, selective agents is currently underway. Most of the compounds tested have been nucleoside analogs which have targeted HCMV DNA polymerase. Several have been used for treating HCMV infections, with phosphonoformate and ganciclovir being approved for clinical use (Balfour, 1990). Recently, several other candidates for treating HCMV disease have been studied, including HPMPC ((*S*)-1-(3-hydrox-2-phosphonylmethoxypropyl)cytosine) (Neyts et al., 1990), BHCG ((±)-(1a,2(ß,3a)-9-[2,3-bis(hydroxymethyl)cyclobutyl]-guanine, SQ 33054) (Field et al., 1990) and SR 3773 (9-[*R*(-1)-1-hydroxymethyl-3-phosphsono)propyloxy methyl]guanine) (Smee et al., 1994). Ganciclovir, however, remains the drug of choice for most life-threatening infection and has been approved for routine use against HCMV retinitis. However, there are major problems with prolonged ganciclovir treatment which is often required to treat HCMV infections. The drug is ineffective against latent infections; prolonged therapy is required to keep the virus infection quiescent. Prolonged therapy often results in bone marrow toxicity (Meyers, 1991). In addition, resistance to ganciclovir also develops during prolonged drug therapy (Drew et al., 1991).

As research with anti-HIV compounds expanded to evaluate non-nucleoside types of antiviral agents, it was found that anthraquinones and anthraquinone-like compounds, such as hypericin, represented a new class of potential antiviral agents (Nakashima and Yamamoto, 1987; Meruelo et al., 1988; Schinazi et al., 1990; Tang et al., 1990; Anderson et al., 1991; Hudson et al., 1991). Some of these compounds were also been found to inhibit herpes simplex viruses (Lavie et al., 1987) and Epstein–Barr virus (Konoshima et al., 1989). As a result of these studies, a number of anthraquinone and anthraquinone-like compounds were tested for non-virucidal antiviral activity against HCMV (Barnard et al., 1992). In those experiments, many of the active anthraquinones were substituted at the C1, C2, C3 and C4 positions with hydroxyl groups. In addition, many of those active anthraquinones in the study were not very soluble in aqueous medium. Addition of sulfonate groups to the basic ring structure or to side chain substitutions renders some anthraquinones more soluble in aqueous media (Green, 1990). Because of this increased solubility and the recent reports that some sulfonated dyes have been found to be active against HIV (Clanton et al., 1992, Schinazi, et al., 1990), consideration has been given to evaluating sulfonated anthraquinones and derivatives against HCMV.

This report describes the in vitro antiviral activity of C1, C2, C3, and C4 substituted anthraquinones and sulfonated derivatives of anthraquinones against HCMV in vitro and introduces a new method for determining the DNA damage potential of antiviral compounds. Structure–activity relationships are also discussed.

2. Materials and methods

2.1. Compounds

All test compounds evaluated in this study were obtained from Aldrich Chemical, Inc. (Milwaukee, WI) except for solway purple, alizarin viridin and solway blue SE which were obtained from Pfaltz and Bauer (Waterbury, CT) and alizarin sodium

sulfonate (alizarin Red S), alizarin complexone and alizarin blue black B which were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cells and virus

Human foreskin fibroblasts were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were routinely grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 4.5 mg/l glucose, 10% fetal bovine serum and 0.1% $NaHCO_3$ without antibiotics. A-549 cells, derived from a human lung carcinoma, were obtained from ATCC and grown in DMEM supplemented with 4.5 mg/l glucose, 0.1% $NaHCO_3$ and 10% fetal bovine serum. Raji cells, a human lymphoblast cell line (ATCC), were maintained in RPMI 1640 supplemented with 10% FBS (Hyclone, Logan, UT) by subcultivation at least twice a week. HCMV, strain AD169, was obtained from ATCC and the EC strain was a gift from Dr. Earl Kern (University of Alabama-Birmingham, Department of Pediatrics and Microbiology, Birmingham, AL).

2.3. Virucidal assay

The method of Barnard et al. (1992) was used. Each test compound was diluted to various concentrations and each concentration was mixed with an equal volume of virus having a titer of 1×10^5 plaque-forming units/ml and incubated at 37°C for 1 h. Surviving virus was assayed by cytotopathic (CPE) assay. Each concentration of compound was assayed in quadruplicate. The concentration of compound that reduced virus titer by 99% (EC_{99}) compared to untreated, controls was then calculated.

2.4. CPE inhibition assay

The in vitro antiviral assay was done as described by Barnard et al. (1992) and modified for HCMV. Cells were seeded at 5×10^3 cells/well in a 96-well plate and incubated at 37°C until confluent (usually 24–36 h). The medium was removed from the cells and varying concentrations of test compound (half log dilutions beginning with 1000 μg/ml) in MEM without serum were added to the appropriate wells. Each concentration was plated 4–6 times per experiment. An equal volume of MEM supplemented with 5% serum and containing virus at 1000 cell culture 50% doses ($CCID_{50}$)/ml was added to the appropriate wells. The plate was incubated at 37°C until virus controls approached 100% viral cytopathic effects. Each concentration of compound was assayed in triplicate. Uninfected, untreated cell controls and untreated, infected controls were included in the assay. Fifty percent effective concentrations (EC_{50}) were calculated by regression analysis using the means of the CPE ratings at each range of compound. Morphological changes due to compound cytotoxicity were graded on a scale of 0–5 with a grade 5 being defined as complete cytotoxicity and compound with uninfected control cells. The 50% cytotoxic dose (IC_{50}) was calculated using regression analysis. A selective index (SI) was calculated for each compound using the formula: $SI = IC_{50}/EC_{50}$. Compounds with very little activity ($SI < 6$) were assayed only one time.

2.5. Plaque reduction assay

Assays were done as described by Barnard et al. (1992) using human foreskin fibroblasts cells and 100 (PFU) of virus/well of the appropriate strain of HCMV. The plates were centrifuged at 2200 rpm for 30 min at room temperature to facilitate virus adsorption (Gleaves, 1984). Morphological changes due to compound cytotoxicity were graded as described below prior to fixing and staining of monolayers. Each concentration of compounds was assayed in triplicate. EC_{50} and IC_{50} values were calculated by regression analysis.

2.6. Determination of cytosolic lactate dehydrogenase activity (LDH)

Cells were seeded at 1×10^4 cells/well in 96-well plates and allowed to attach for 4 h. Growth medium was then removed and replaced with medium containing the appropriate concentration of dye. To determine the effects of the compounds on membrane permeability, cells treated with the dyes were then assayed for a cytosolic enzyme, LDH. After 24 h, cells were assayed for LDH activity using a 'Cytotox 96 Assay®' kit (Promega, Madison WI). Briefly, medium was aspirated from each well plate and the plates rinsed $1 \times$ with MEM. To each well, 180 μl of MEM without serum was added along with 20 μl of the $10 \times$ lysis solution provided with the kit. The cells were incubated at 37°C for 45 min and then centrifuged 4 min at 250 g. Aliquots (50 μl) of supernatant fluid were transferred to a flat-bottomed 96-well plate and assayed for LDH as outlined in the instructions of the kit. Absorbance at 490 nm was read using Automated Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). A control well containing a known amount of LDH was used as a positive control and a well containing MEM and lysis solution was used as a negative control. Each concentration of drug was assayed in quadruplicate. The concentration of LDH assayed in cells at each dosage of compound was expressed as average percent of untreated control cells.

2.7. Neutral red assay for toxicity

A modified method of Cavenaugh et al. (1990) was used. Test compounds at varying concentrations were added to near confluent cell monolayers and incubated at 37°C for 7 days. Appropriate untreated controls were also included in the assay. Medium was removed and 0.2 ml of neutral red (0.034% in PSS) was added to each well and incubated for 2 h at 37°C in the dark. The neutral red solution was then removed from each well and the wells rinsed two times with PBS (pH 7.4). Equal volumes of absolute ethanol and Sörenson citrate buffer (0.1 M sodium citrate, 0.1 M HCl, pH 4.2) were mixed together and added to each well. Plates were incubated in the dark for 30 min at room temperature to solubilize the dye. The plates were then gently mixed on a 96-well plate adapted vortexer for 1 min. Absorbance at 540 and 450 nm was read with a microplate reader. All compound concentrations were assayed in quadruplicate. Absorbance values were then expressed as percents of untreated controls and IC_{50} values were calculated by regression analysis.

2.8. Log phase cell toxicity assay

Cells were seeded in 48-well plates at 1×10^4 cells per well and allowed to adhere during a 3-h incubation period at 37°C. Varying concentrations (in duplicate) of test compound in MEM supplemented with 10% FBS or MEM-10% FBS without compound were added to each well. Cells were incubated at 37°C until monolayers were formed in control wells ($\approx$ 18 h). Cells from each well were stained with trypan blue, counted in a Coulter Counter (Coulter Corp., Miami, FL) and viable cell counts were expressed as percent of control. The IC_{50} values were derived from the averages of three samples of each concentration were calculated by regression analysis when possible.

2.9. Biochemical cytotoxicity assay

The assay for quantitating the uptake of radiolabeled macromolecular precursors in the presence or absence of compound was done as described by Barnard et al. (1992). The effects of each test compound on the uptake of [^{3}H]uridine (to measure effects on RNA synthesis), [^{3}H]leucine (to measure effects on protein synthesis), and [^{3}H]thymidine (to measure the effects of DNA synthesis) into acid-soluble and -insoluble fractions of late-log phase human diploid cells (Hs-68) were determined following an 18-h incubation period with drug and subsequent 1-h pulse period with the appropriate macromolecular radiolabeled precursor in the presence of fresh antiviral compound. All statistical comparisons were made by analysis of variance. The experiment was done one time and each treatment was assayed in quadruplicate.

2.10. Assessing DNA damage by sulfonated anthraquinones

Cells were exposed to the test compounds at various concentrations for an 18-h period at 37°C. After the exposure time, cells were washed several times in PBS and resuspended PBS at a final concentration sufficient to provide approximately 10,000 cells per slide for DNA damage analysis. Cell viability was also concurrently determined by trypan blue exclusion.

The comet assay was performed essentially as described previously (O'Neill et al, 1993) with some minor modifications. Raji cells exposed to various concentrations of the test compounds were suspended in low melt point agarose to a final concentration of 0.75% and spread evenly over a fully frosted slide. The slides were sequentially placed in lysis solution (0.03 M NaOH, 1.0 M NaCl, 0.5% Na-lauroyl sarcosine) for 1 h and a wash solution (0.03 M NaCl, 2 mM EDTA) for another hour to lyse the cells and allow unwinding of the protein-stripped DNA and expression of DNA damage. Slides were electrophoresed for 10 min at 1.71 V/cm, neutralized, and stained with ethidium bromide for comet visualization.

Individual cells were examined for DNA damage using a Zeiss epifluorescence microscope attached to an MTI intensified solid-state CCD camera and an ITI ITEX image analysis system. Comets were viewed using a 32 $\times$ objective and projected to a 17-in. Toshiba monitor for analysis of DNA migration. Images were selected randomly from the center of the slides. We used a comet image analysis program written by Dr.

Ralph Durand (Olive et al., 1990) in order to measure comet tail moment, which is the most descriptive metric of DNA damage using the single cell gel assay system. Tail moment is described as the product of the DNA comet tail migration length and the percentage of DNA in the tail. Between 50 and 200 comets were analyzed for each slide. In each experiment the drugs were tested in duplicate, and the data presented are the averages of 3 experiments.

3. Results

3.1. Antiviral activity

A number of sulfonated anthraquinones and anthraquinone derivatives were tested for antiviral activity (Tables 1 and 2). If a compound had selective index of less than 6, it was considered inactive (Table 1). Of those compounds tested, 7 had activity against HCMV (Ad 169), including *acid black 48* (1,1'-iminobis(4-amino)9,10-anthracenedione), *acid blue 40* (4-(4-acetamidophenyl-1-amino-9,10-di-oxanthracene-2-sulfonate), *acid blue 129* (1-amino-9,10-dihydro-9,10-dixo-4-(2,4,6-trimethylanilino)anthracene-2-sulfonate), *alizarin violet R* (2,2'-((9,10-dihydro-9,10-dioxo-1,5-anthacene-diyl)di-imino)bis(5-methyl-,disodium salt)), *reactive blue 2* (1-amino-4-((4-((4-chloro-6-

Table 1
Anthraquinones and derivates found to have minimal HCMV (AD-169) inhibitory activity (SI < 6)

Compound	EC_{50} [a]	IC_{50} [b]	SI [c]
Alizarion viridin	50	100	2
Remazol brilliant blue R	10	20	2
Uniblue A	110	250	2
Acid blue 25	40	60	1
Acid green 27	20	20	1
Solvent blue	30	30	1
Acid blue 41	> 200 [d]	265	< 1 [e]
Acid blue 45	> 100 [d]	120	< 1 [e]
Acid blue 80	> 200 [d]	590	< 1 [e]
Acid blue black B	> 50 [d]	50	< 1 [e]
Alizarin complexone	> 100 [d]	170	< 1 [e]
Alizarin red S	> 100 [d]	110	< 1 [e]
Basic blue 47	> 10 [d]	10	< 1 [e]
Oil blue N	> 260 [d]	150	< 1 [e]
Solvent blue 59	> 10 [d]	20	< 1 [e]
Solvent green 3	> 2 [d]	10	< 1 [e]
Solway blue SE	60 [d]	15	< 1 [e]

[a] The concentration of compound (μM) that reduces virus cytopathic effect by 50%.
[b] The 50% cytotoxic dose as determined by light microscopy.
[c] Selective index (IC_{50} /EC_{50}).
[d] Virus cytopathic effect could not be distinguished from compound cytopathic effects at concentrations greater than that listed.
[e] Values less than 1 indicate a lack of antiviral selectivity.

Table 2
Effects of active anthraquinones and derivatives (SI ⩾ 6) on the replication of HCMV AD-169 and a ganciclovir-sensitive clinical isolate, strain EC [a]

Compound	AD-169			Strain EC		
	EC_{50} [b]	IC_{50}	SI	EC_{50} [b]	IC_{50}	SI
Acid black 48 [c]	30	> 1500 [d]	> 50	75	> 1500 [d]	> 20
Acid blue 129	7	275	40	15	275	18
Reactive blue 2	4	150	38	20	150	8
Acid blue 40	10	380	38	20	380	19
Alizarin violet R	10	300	30	50	300	6
Reactive blue 4	30	280	9	15	280	19
Solway purple	150	930	6	120	930	8

[a] Values represent the average of two experiments.
[b] Concentration of compound (μM) that reduces plaque production (EC virus assay) or virus cytopathic effect (AD-169 assay) by 50%.
[c] Acid black 48 is complexed with an undefined number of SO_3Na groups. Therefore, the formula weight used was assuming an average of 2 sulfonate groups.
[d] Little or no toxicity detected at the highest concentration tested. Therefore an IC_{50} could not be calculated.

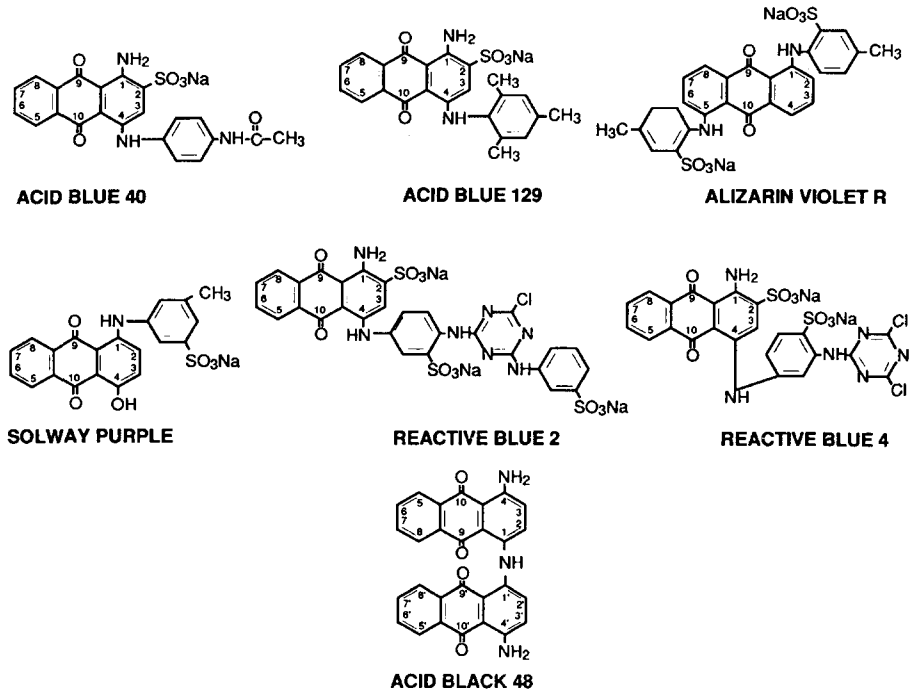

Fig. 1. Structures of active anti-HCMV anthraquinones. The numbering system is used in the figure is for the sake of comparison and is not necessarily consistent with the nomenclature found in Chemical Abstracts or with IUPAC nomenclature.

((4)-sulfophenyl)amino)-1,3,5-triazin-2-yl)amino)-3-sulfophenyl)-amino)-9,10-dioxoanthracene-2-sulfonic acid), *reactive blue 4* (1-amino-4-(2'-(4,6-dichloro-*s*-triazin-2-ylamino)phenylamino)-9/10-dihydro-9/10-dioxoanthracene-2,4'-disulfonic acid), and *solway purple* (sodium 4-((9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthryl)amino) toluene-3-sulfonate), (Table 2). Five of the compounds were sulfonated derivatives of anthraquinones. Four of those were substituted at C1, C2 and/or C4 positions. A sulfonate group found at the two position, solway purple (SP) and alizarin violet R (AVR) being the exceptions (Fig. 1). The other active compound, acid black (48 ABL-48), was not sulfonated and is an anthrone resembling a known active anti-HCMV anthraquinone derivative, hypericin (Barnard et al., 1992). AVR, ABL-48, acid blue 40 (AB-40), acid blue 129 (AB-129) and reactive blue 2 (RB-2) had the most preferential anti-HCMV activity with selective indices of 30 or greater. Ganciclovir had a selective index of > 750. RB-2, AB-129, AVR and AB-40 were the most potent, with EC_{50} values of 10 μM or less. Ganciclovir had an EC_{50} of 10 μM. The virucidal concentrations required to inactivate 99% of the infectious virus inoculum were well above the EC_{50} values for HCMV inhibition (AVR = 1000 μM, ABL-48 > 1000 μM, AB-40 > 2110 μM, AB-129 > 2180 μM, RB-2 > 1190 μM, reactive blue 4 (RB-4) > 1570 μM, SP > 2220 μM). Ganciclovir was active at 5 μM.

When the active compounds were evaluated for activity against a clinical isolate of HCMV (Table 2), all compounds were less active against this isolate compared to their efficacy against HCMV Ad-169 (see EC_{50} values, Table 2). An exception to this was RB-4, which appeared to be twice as inhibitory to the clinical isolate and SP which had equivalent activity against both virus strains.

3.2. Cytotoxicity

There has been some concern about the potential toxicity of these types of compounds. Therefore, we decided to determine their toxicity by a variety of methods

Table 3
Toxicity of active anti-HCMV anthraquinones in log phase human foreskin fibroblasts (Hs-68) and in human lung epithelial cells (A549)

Compound	Cell viability		Neutral red uptake	
	Hs-68 cells (IC_{50}) [a]	A-549 cells	Hs-68 cells (IC_{50}) [a]	A-549 cells
Acid black 48	$> 500 \pm 20$ [b]	500 ± 40	$> 1500 \pm 100$	$> 1500 \pm 50$
Acid blue 40	$> 2000 \pm 100$	$> 2000 \pm 70$	1350 ± 60	1880 ± 50
Acid blue 129	380 ± 40	320 ± 60	$> 2000 \pm 70$	$> 2000 \pm 120$
Alizarin violet R	$> 500 \pm 40$	280 ± 30	$> 1500 \pm 150$	390 ± 40
Reactive blue 2	$> 1000 \pm 60$	130 ± 15	480 ± 70	200 ± 45
Reactive blue 4	$> 1500 \pm 120$	30 ± 6	1570 ± 160	470 ± 20
Solway purple	500 ± 40	730 ± 50	$> 2000 \pm 100$	$> 2000 \pm 80$

[a] Concentration of compound (μM) that reduced the number of cells or neutral red uptake by 50% relative to the cell controls.
[b] Greater than sign indicates that the concentration was the largest dose tested.

Table 4
Effects of sulfonated anthraquinones on the cellular metabolism of actively growing cells

Compound	Inhibition of macromolecular synthesis in Hs-68 Cells (IC_{50})			Inhibition of lactate dehydrogenase (IC_{50}) [a]	
	DNA	RNA	Protein	Hs-68 cells	A-549 cells
Acid black 48	> 1500 ± 150 [a]	500 ± 20	> 1500 ± 100	> 1000 ± 60 [a]	50 ± 3
Acid blue 40	200 ± 10	> 2000 ± 120	> 2000 ± 180	> 2000 ± 30	230 ± 10
Acid blue 129	1100 ± 100	> 1000 ± 100	1000 ± 150	> 2000 ± 25	160 ± 25
Alizarin violet R	60 ± 20	> 1500 ± 80	1600 ± 110	> 1500 ± 210	170 ± 20
Reactive blue 2	> 1000 ± 90	1200 ± 95	20 ± 3	> 1000 ± 30	100 ± 5
Reactive blue 4	> 1500 ± 120	> 1500 ± 85	20 ± 2	> 1500 ± 45	70 ± 6
Solway purple	1800 ± 160	> 2000 ± 210	> 2000 ± 160	> 1000 ± 35	310 ± 15

[a] Greater than sign indicates that the concentration was the largest dose tested and therefore, no IC_{50} values could be calculated.

(Tables 3–5). The viability of cells after a short-term exposure (24 h) to a compound following cell adherence was compared in two different cell lines (Table 3). The compounds were not very toxic to human diploid foreskin fibroblasts, but showed variable toxicity to A-549 cells. RB-4 was relatively toxic to actively growing A-549 cells (IC_{50} = 30 μM). The order of least toxicity in A-549 cells was AB-40 ≅ ABL-48 < AB-129 < AVR < RB-2 < RB-4.

We also evaluated the effects of these compounds on various aspects of cellular metabolism in actively growing cells. AVR and AB-40 inhibited DNA synthesis at 60 and 211 μM, respectively (Table 4). None of the compounds dramatically inhibited RNA synthesis. AB-129 and AVR only slightly inhibited protein synthesis, probably due to perturbation of the soluble pools of amino acids (at least leucine). IC_{50} values for inhibition of the uptake of radiolabeled into acid-soluble pools was 574 μM for AB-129 and 495 μM for AVR.

The amount of lactic acid dehydrogenase activity, which reflects mitochondrial activity in cells, was measured by quantitating the release of lactic acid dehydrogenase from cells lysed with detergent (Table 4) after 24 h exposure of freshly seeded cells to the compounds. Again HS-68 cells appeared to be much less sensitive to the toxic effects of these compounds than A-549 cells. ABL-48 appeared to be the greater inhibitor of the total amount of LDH found in A-549 cells when compared with the other compounds.

3.3. Genotoxicity

Of great concern with any anthraquinone containing amino groups is the carcinogenic, mutagenic potential and genotoxic effects of such compounds (Venturini and Tamaro, 1979). Therefore, we measured the ability of these compounds to cause DNA strand breaks (Table 5) by comet assay. In this assay the most descriptive metric of DNA damage is measuring the comet tail moments which is the product of the DNA comet tail migration length and the percentage of DNA in the tail (Olive et al., 1990). A tail moment of greater than 2 is considered positive for significant DNA damage. With a tail moment of 2 at 30 μM, RB-2 was the most genotoxic of the compounds tested.

ABL-48 had a tail moment of 2 or greater at concentrations $\geqslant 170$ μM. AVR and SP were not genotoxic at the concentrations tested.

4. Discussion

Previous work (Tang et al., 1990, Schinazi et al., 1990; Barnard et al., 1992) have indicated that anthraquinones have anti-HIV and anti-HCMV activity. Some of these compounds also inhibited ganciclovir-resistant HCMV (Barnard et al., 1992). Of those compounds that were active, many of them had hydroxy substitutions at the C1, C2 and C4 positions. In the present study we wanted to determine if other group substitutions found in commonly available anthraquinone dyes would render a compound more or less active against HCMV. Substitution with an aliphatic side chain at C3 resulted in a compound of much less solubility and lower antiviral activity. Adding a sulfonate group at C3 to increase solubility 50-fold (data not shown) also resulted in much less anti-HCMV activity.

Some structural activity relationships could be established from the data presented in this study. Barnard et al. (1992) reported that simple hydroxy-substituted anthraquinones were the most active against HCMV, the most common substitutions occurring at C1, C2 and C4. In the present study, we found that a substitution of an aliphatic side chain at C3 on alizarin to get alizarin complexone resulted in complete loss of antiviral activity, probably because the aliphatic side chains rendered the compound much less soluble in aqueous medium. Sulfonating the alizarin structure at C3 (alizarin sulfonate, alizarin red S) enhances the water solubility of alizarin by 50-fold, nevertheless the substitution resulted in an inactive compound.

Compounds with substitutions at C1 (NH_2), C2 ($NaSO_3$) and C4 (heterocyclic rings with side groups) of the basic anthraquinone structure were very active (AB-129 and AB-40). However, if the heterocyclic phenol group had no substitutions (acid blue 25), it had very little antiviral activity and a great deal of toxicity. If the sulfonate group at C2 was eliminated, but the substitutions at C1 and C4 remained as described above (basic blue 47 and solvent green 3), antiviral activity was not detected because of extreme toxicity. When the substitution at C4 was an aliphatic side chain (oil blue N, solvent blues 35 and 59) in lieu of a heterocyclic moiety, the anti-HCMV activity was also dramatically reduced. An additional methyl group on the heterocyclic ring added at C4 to get acid blue 41 from acid blue 40 resulted in a total loss of antiviral activity. Interestingly, if the sulfonate on the heterocyclic ring was oxidized to SO_2^- by addition of an aliphatic group, the compound was either not active (Uniblue A) or very toxic (Remazol brilliant blue R). All compounds that either had straight chain additions or heterocyclic additions to both of the amine groups linked to C1 and C4 (acid green 27, solvent blue 35, oil blue N, solvent blue 35 and solvent green 35) were inactive and/ or cytotoxic.

A symmetrical anthraquinone such as acid blue 45 with sulfonate groups at C2 and C6; hydroxyl groups at C1 and C5, and amino groups at C4 and C8 and no heterocyclic ring as substitutions was inactive, whereas a symmetrical anthraquinone like AVR with sulfonated heterocyclic groups at C1 and 5 was highly active.

A troublesome problem with anthraquinones is their lack of solubility. Adding sulfonate groups to anthraquinones and anthraquinone derivatives increases their solubility (Green, 1990). Therefore, one objective of the present studies was to determine if sulfonated anthraquinones with presumably enhanced solubility would result in a more active anti-HCMV compound. Two structural groups of compounds were found to be active. In one group of compounds tested, all were substituted at C2 with a sulfonate group and substituted at C4 with a heterocyclic ring(s) linked to the basic anthraquinone structure by an amino group. Many of the heterocyclic rings were also substituted with sulfonates. The second group consisted of one compound, AVR, which was symmetrically arranged with sulfonated heterocyclic phenol groups linked by an amide to C1 and C5. A third structurally active group without sulfonates was also found to have potent anti-HCMV activity, ABL-48. It has a dimer structure similar to a known active anti-HCMV agent, hypericin.

Four compounds were evaluated for genotoxic effects using a unique assay for evaluating DNA strand breaks, the comet assay. Surprisingly, acid black 48 showed some ability to cause DNA strand breaks although it was found to be relatively non-cytotoxic by other assays. RB-2 was also found to cause genotoxicity, although Venturini and Tamaro (1979) found that RB-2 was negative in the Ames' *Salmonella typhimurium* test for mutagenic potential. Our results are in agreement with Brown and Brown (1976) who found that anthraquinones having sulfonate or akyl substitutions were non-mutagenic, although verification of this lack of DNA damaging effect should be verified in other assays.

Most of the compounds showed variable toxicity in the two cells lines in which several aspects of cytotoxicity were evaluated. A-549 cells were much more sensitive to the anthraquinones than Hs-68 cells. Interestingly, both reactive blue compounds inhibited protein synthesis which parallels the commercial usage of RB-2 as a protein binding agent (RB-2 = Cibracon® F3GA) for affinity chromatography.

If all the compounds are ranked for each cytotoxic or antiviral assay for being least toxic or for having the most potent antiviral activity, acid blue 129 appeared overall to be the most effective antiviral compound tested followed by acid black 48, acid blue 40, alizarin violet R and solway purple. Reactive blue 2 and 4 were the least effective anti-HCMV compounds, mainly due to toxicity. Solway purple, though not very toxic, was almost inactive against HCMV. Not surprisingly, the compounds were not quite as effective against a clinical isolate of HCMV, probably due to the fact that the AD169 laboratory strain has become attentuated because of many years of passage in cell culture.

The potential mechanism of inhibition could include abrogation of the virus envelope-cell membrane fusion process as has been shown with other sulfonic acid dyes (Clanton, 1992). In addition, some anthraquinones have been shown to intercalate in to DNA (Lown, 1993; Patterson, 1993). The compounds may intercalate viral DNA much more readily than host cell DNA while viral DNA is being actively replicated. In support of this is the lack of genotoxicity in a comet assay and the lack of inhibition of DNA synthesis by any of the active compounds. However, most anthraquinones shown to intercalate DNA have acidic hydroxyl groups at the C1 and/or C8 positions (i.e. hypericin and dianthrol (Muller, 1988; Patterson, 1993)).

Non-specific attachment of dyes to viral proteins or virus receptors on host cells could also be a mechanism. The reactive blue compounds, RB-2 for instance, binds protein, such binding might then block initial virus–cell interactions.

Since there is a need for alternative anti-HCMV compounds with novel targets of inhibition, some of the active anthraquinones in this study may serve as model compounds for future compound alternative therapies for HCMV infections that could be used.

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