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Herpes simplex type 1 : *lac*Z recombinant viruses. II. Microtiter plate-based colorimetric assays for the discovery of new antiherpes agents and the points at which such agents disrupt the viral replication cycle

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Abstract

A panel of microtiter plate-based colorimetric assays for monitoring HSV-1 growth has been made. The panel consists of 4 different HSV-1 (strain KOS) *lac*Z recombinant viruses which express β-galactosidase under the control of different HSV-1 promoters derived from each class of herpes simplex gene expression: immediate–early (ICP4), early (TK), delayed early (gD) and late (gC). Inhibitors of HSV-1 growth were evaluated using differential effects on each of the reporter viruses as a measure of which points in the viral replication cycle an inhibitor was acting. Aphidicolin (DNA synthesis inhibitor) was studied as a model compound. At an m.o.i. of 0.05, at 24 h postinfection (h p.i.), aphidicolin inhibited 80% of viral growth at 1 μg/ml, as determined by a reduction in ICP4-driven activity within the second cycle of infection. At m.o.i. 5, within the first infectious cycle, aphidicolin had no effect on the signals from either the ICP4 or TK viruses at 3 μg/ml, while largely suppressing gD and fully inhibiting gC-driven signals at 2 μg/ml. This profile is consistent with the behavior expected of a DNA synthesis inhibitor. Five inhibitors of unknown mechanism were evaluated. Two compounds inhibited ICP4-driven activity within the first infectious cycle and were classified as potential inhibitors of viral entry, uncoating or IE gene expression (XF884, BT318). One compound inhibited gD and gC-driven activity without inhibiting signal from the ICP4 and TK viruses, and was classified as a potential DNA synthesis

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inhibitor (DS810). Two compounds (S5193, ER622) had effects on gD- and gC-driven activity which were somewhat different from aphidicolin and DS810, but which could be interpreted as inhibition of viral assembly and/or egress. The potency of XF884 varied with the time postinfection at which it was added to cells (IC_{50} 3.7 to > 10 μg/ml) while the effects of BT318 were independent of time of addition (IC_{50} 11.4 μg/ml). These results suggest XF884 inhibits viral entry while BT318 is acting after viral entry, possibly as a direct inhibitor of ICP4 gene expression. Together, these results suggest the panel of recombinant herpes viruses has utility in aiding in the identification of the points in the herpes life cycle at which antiherpes drug candidates, of unknown mechanisms, are acting.

Keywords: Herpes simplex virus type 1 (HSV-1); *lac*Z; Recombinant herpes simplex virus type 1

1. Introduction

Current cell-based (live virus) screens for detecting antivirals require multiple cycles of virus replication in order to observe output (plaque, yield reduction, CPE assays). The outputs in these screens reflect the cumulative biology of many individual virally directed processes – entry, uncoating, transcription, translation, protein processing, transport, and viral egress. In an effort to analyze the mechanisms of action of characterized and uncharacterized inhibitors of HSV-1 growth, we have created a family of HSV recombinant viruses which express β-galactosidase as a function of each of the individual temporal classes of HSV genes (immediate–early, early, delayed early and late) (Dicker and Seetharam, 1995). The sensitivity and format of these assays is close to that of standard plaque assays, but differs somewhat in that the measured signal is cumulative. In this respect, these assays can be thought of as sensitive, semi-quantitative CPE assays. In this paper we report the application of this panel to investigate the mechanisms of action of newly discovered antiherpes compounds.

2. Experimental

2.1. Materials and methods

African green monkey (Vero) cells were obtained from ATCC (CCL 81). Cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Gibco), penicillin–streptomycin (Gibco) and 1 mM glutamine (Gibco). HSV-1 (strain KOS) was obtained from Dr. Priscilla Schaffer via Dr. Annie Colberg-Poley and propagated according to standard protocols. β-Galactosidase assays for transient transfections and infections were performed according to the method of Eustice et al. (1991), using chlorophenol red-β-D-galactopyranoside (CPRG) (Boehringer-Mannheim) as substrate. HSV-1 (strain KOS) recombinant viruses ICP4*lac*ZB, TK*lac*Z and gD*lac*ZA (Dicker and Seetharam, 1995) were used. An HSV recombinant virus in which the expression of β-galactosidase was under the control of the HSV-1 late gene gC regulatory region (gC*lac*Z) was a kind gift from Dr. Jerry Weir, Walter Reed Army Institute of Research.

2.1.1. Microtiter plate assays

Ninety-six-well microtiter plates (Corning no. 25860) were seeded with Vero cells (3×10^4 cells in 25 μl/well) and incubated overnight at 37°C. Medium was removed and cells were infected with virus (in 25μl of medium) at the appropriate m.o.i. The particular m.o.i. for each virus was defined by that m.o.i. which gave a high level of β-galactosidase activity at the defined period of viral growth for that particular assay. In practice, this m.o.i. was the minimal m.o.i. capable of eliciting at least a 50-fold increase of β-galactosidase activity over background within that time course. Except for evaluation of XF884, after 1.5 h at 37°C, 25 μl of fresh medium, or medium containing the test compound at twice the final concentration, was added and the cells were incubated further at 37°C. For XF884, virus was added to cells by admixing virus in 25 μl medium plus 25 μl of a 2 × stock of XF884; medium was not removed, in contrast to the other tested compounds. At the desired time postinfection, 50 μl of β-galactosidase lysis buffer containing CPRG (final concentration 0.4 mg/ml) was added and the extracts were mixed on a plate shaker for 1 min. β-Galactosidase activity was monitored by the change in absorbance at 575 nM on a Molecular Devices Thermomax microtiter plate reader at room temperature. All assays were done in quadruplicate unless noted otherwise, with β-galactosidase activities in drug-containing wells normalized to no-drug control wells.

3. Results

3.1. Using the panel of herpes viruses to study the mechanism of action of aphidicolin

The recombinant viruses and assay types used in this study are listed in Table 1. The assays were designed to work within 96-well microtiter formats. A different set of

Table 1
The 4 colorimetric assays and information implied by β-galactosidase expression

Virus	Temporal class	m.o.i.	Hours [a]	Summary of effects [b]
ICP4*lac*ZB	Immediate early (IE)	5	6.5	Effects on entry and/or IE synthesis
TK*lac*Z	Early (E)	10	6.5	Effects on entry + IE + E synthesis
gD*lac*ZA	Delayed early (DE)	5	24	Effects on entry + IE + E + DE synthesis
gC*lac*Z	Late (L)	5	24	Effects on entry + IE + E + DE + L synthesis
ICP4*lac*ZB	Assembly and egress (AE)	0.05	24	Effects on entry + IE + E + DE + L synthesis + effects on assembly and/or egress

[a] Time before β-galactosidase activity determined.
[b] Summary of effects on β-galactosidase expression.

conditions (m.o.i., hours postinfection (h p.i). for measurement of β-galactosidase activity) was employed for each assay so that the signal-to-noise ratio would be > 50. When run in a format with a high (m.o.i. 5) viral challenge, and measured at 5 h p.i., the ICP4*lac*ZB virus provides a measure of IE gene expression which has occurred after viral entry, but prior to viral replication. When used in a format with a low viral challenge (m.o.i. 0.05), and measured at 24 h p.i., the assay reports IE gene expression derived from reinfection by the ICP4*lac*ZB virus released after the first round of replication. Second cycle kinetics for the ICP4*lac*ZB virus are as described (Dicker and Seetharam, 1995) and coincided with an 18- to 22-h cycle (Fields and Knipe, 1990). Expression in this latter format (AE) includes all of the events from first cycle entry through second cycle re-entry and is thus a surrogate for a plaque assay. Intermediate points of the cycle are monitored by TK*lac*Z, gD*lac*ZA and gC*lac*Z. The TK*lac*Z virus provides a measure of cumulative entry, IE and E gene expression when used with a high viral challenge (m.o.i. 10) and measured at 5 h p.i. The gD*lac*ZA and gC*lac*Z viruses give a cumulative report of events up to and including DE and L gene expression when run at high viral challenge and assayed at 20 h p.i. Because of the sequential nature of the viral program, inhibition of entry or immediate–early synthesis ought to exert cumulative effects on early, delayed early and late gene expression. However, inhibitors which act at later stages should have no effect on earlier reports. For example, inhibition of DNA synthesis should have no effect on the IE and E reports, but large effects on the DE, L and AE reports. The goal of this series of experiments was to monitor the expression of β-galactosidase within the first and second cycles in order to obtain information about which points in the viral replication cycle a particular antiherpes compound may be acting. To validate the method, we used aphidicolin as a positive control, since it is known that aphidicolin acts by inhibiting DNA synthesis (Spadari et al., 1985). The temporal class (IE, E, DE, L, AE) is also listed in Table 1, along with a summary of the modes of inhibition which are implied by the inhibition of β-galactosidase expression observed for each particular class.

Monolayers of Vero cells in 96-well microtiter plates were infected with virus with or without aphidicolin and subsequently assayed for β-galactosidase activity, as described in Materials and methods. The results of this experiment are shown in Fig. 1.

Aphidicolin had no effect on the expression of β-galactosidase from the ICP4 and TK viruses (IE and E reports), up to a concentration of 3 μg/ml. In contrast, there was an 80% loss of activity from the gD*lac*ZA virus (DE report) and a 95% loss of activity from the gC*lac*Z virus (L report) at 3 μg/ml. The residual activity from the gD*lac*ZA virus is presumably from the early component of gD-driven expression (Johnson and Spear, 1984). Thus, the panel of recombinants correctly characterized the mechanism of action of aphidicolin and thus, the response of the panel can be considered as a prototype for the profile expected of an antiherpes substance acting via inhibition of DNA synthesis.

3.2. Use of the lacZ viruses to determine which points in the viral replication cycle antiherpes compounds are acting

A standard plaque screening approach using HSV-1 (strain KOS) was used to identify compounds which inhibited viral growth. Compounds able to inhibit the growth of HSV

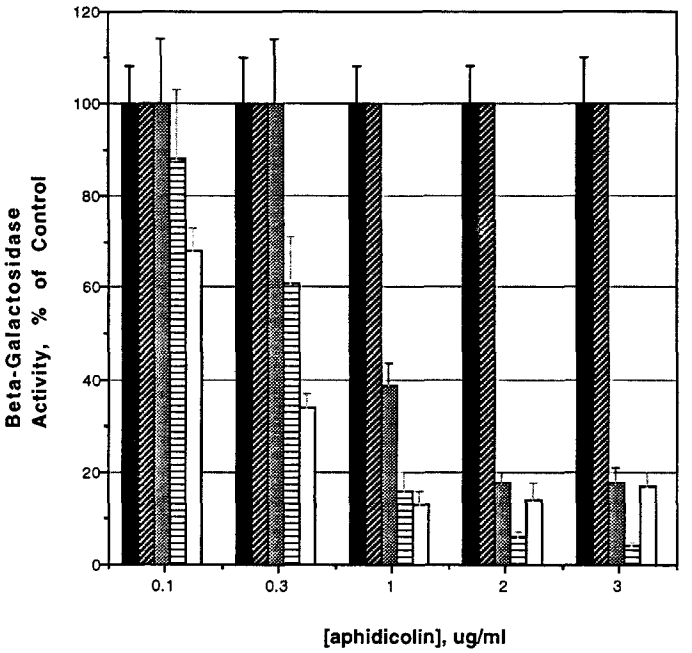

Fig. 1. Effects of aphidicolin on the expression of β-galactosidase by the panel of reporter viruses IE (■), E (□), DE (□), L (□) and AE (□). Monolayers of Vero cells in microtiter wells were infected with the reporter viruses and assayed for β-galactosidase activity, as described in Materials and methods.

by at least 50% at less than or equal to 15 μg/ml were selected for further study (Fig. 2). For comparison, the IC_{50}s for these compounds were also determined using the AE report from the ICP4*lac*ZB virus (Table 2). In general, there was a fair degree of correspondence between the plaque and activity assays. It should be noted that these

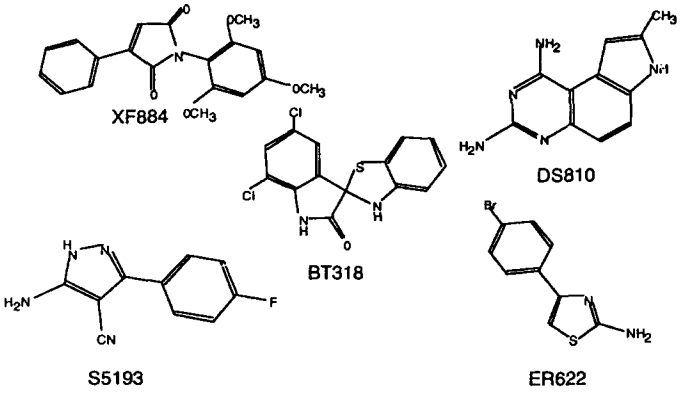

Fig. 2. Structures of compounds which inhibited > 50% of plaque formation of herpes simplex type I (KOS) at concentrations less than 15 μg/ml.

Table 2
Comparison of IC_{50} determinations by plaque versus β-galactosidase assays. Values are in μg/ml.

Compound	IC_{50} [a]	IC_{50} [b]	Toxicity [c]
XF884	3.9	2.3	20
BT318	13.0	7.8	20
DS810	8.3	3.2	20
S5193	3.0	3.0	30
ER622	10.0	8.0	30
Aphidicolin	3	0.2	5

[a] Plaque assays: confluent monolayers of Vero cells were infected with 100 PFU HSV-1 (strain KOS).
[b] β-Galactosidase assays, 3×10^4 cells in microtiter wells were infected with ICP4*lac*ZB at m.o.i. 0.05 and assayed at 24 h p.i., as described in Materials and methods.
[c] Toxicity assays: confluent monolayers of Vero cells were treated with compounds at varying concentrations, without infection. After 48 h, cell viability was determined by staining with crystal violet and noting monolayer coverage. Numbers represent the lowest concentration which showed a cytopathic effect.

compounds are chemically unrelated. They did not directly inhibit the activity of purified β-galactosidase at 30 μM (data not shown). Toxicity was determined by treating confluent monolayers of Vero cells with the compounds at the concentrations used to determine inhibition, but without infection. After 48 h, toxicity was determined by staining with crystal violet and noting monolayer coverage. Therefore, the presumption at the onset of the study was that these compounds might inhibit viral growth by different mechanisms.

Each of the inhibitors was tested with the panel of assays using the same protocol as used for aphidicolin except for XF884. XF884 gave variable results depending on the exact timing on when it was added postinfection. Therefore this compound was added along with virus, whereas the others were added 1.5 h p.i. as described in Materials and methods. The results, including those for aphidicolin, are shown in Fig. 3A–E and are summarized in Table 3, along with an explanation of definitions. Two of the compounds (XF884, BT318) inhibited the IE report. On this basis these compounds were classified as potential inhibitors of viral entry, uncoating or IE gene expression. One compound (DS810) inhibited first-cycle activity from the gD*lac*ZA and gC*lac*Z viruses (the DE and L reports), but did not inhibit either the ICP4 or TK viruses (IE and E reports). This profile was similar to that from aphidicolin, suggesting that DS810 might be a DNA synthesis inhibitor. Two other compounds (S5193, ER622) also inhibited the DE and L reports while having no effects on the IE and E reports. Their profiles, however, differed from the aphidicolin profile. S5193 exerted greater effects on the DE reports than on the L reports at all concentrations tested. For example, at 10 μg/ml, where the AE report was reduced by $> 95\%$, the L report was inhibited by 50% and the DE report by less than 20% By comparison, aphidicolin had larger effects on the L versus the DE reports at all tested concentrations. This suggests S5193 acts late in the viral cycle, but by a mechanism other than DNA synthesis inhibition. Lastly, the profile for ER622 also differed somewhat from aphidicolin in that the DE report was reduced at all concentrations which reduced the AE report. By comparison, there is a concentration range for aphidicolin (0.1–0.3 μg/ml) in which the AE report is reduced while the DE report is unaffected.

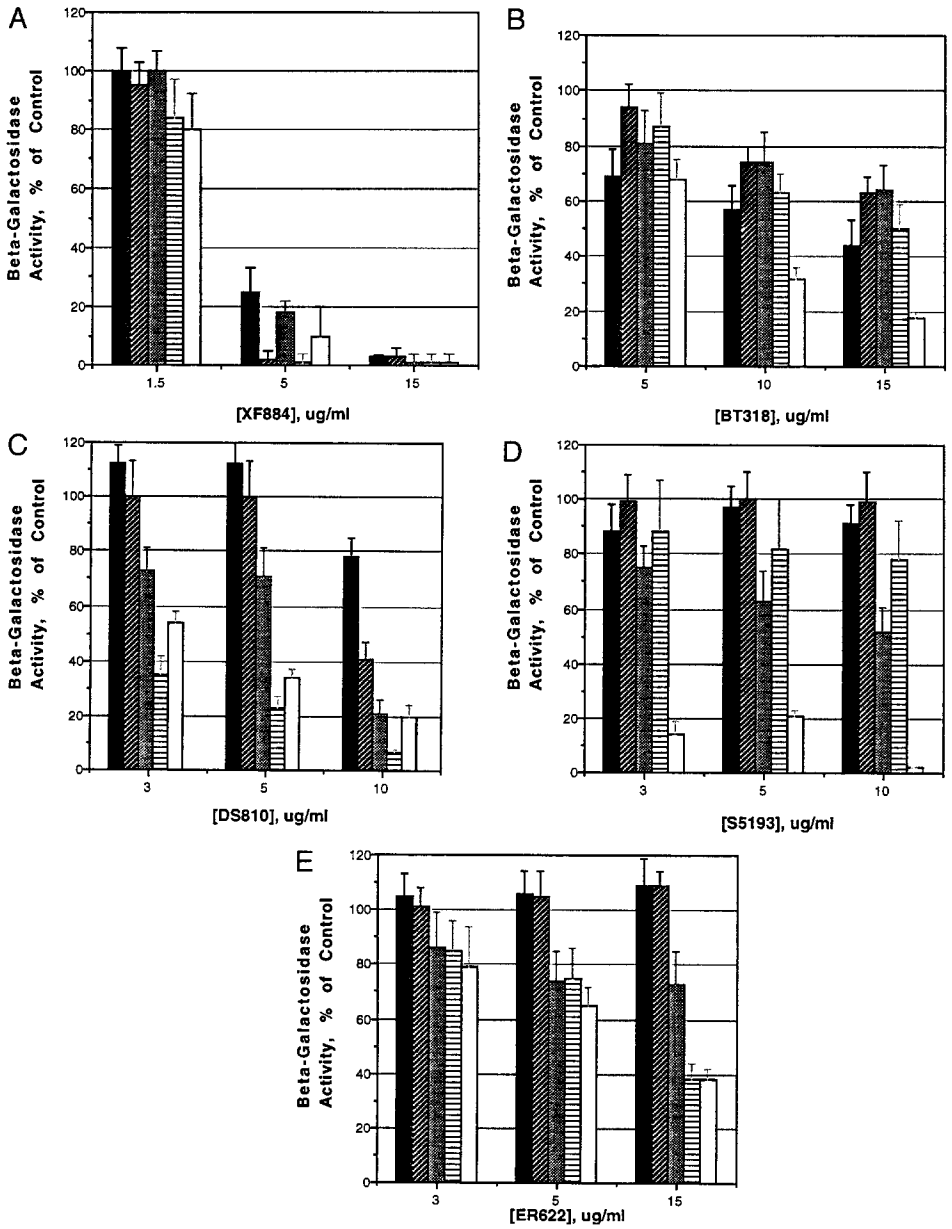

Fig. 3. Effects of antiherpes compounds: XF884 (A), BT318 (B), DS810 (C) S5193 (D), and ER622 (E) on the expression of β-galactosidase by the panel of reporter viruses IE (■), E (□), DE (□), L (□) and AE (□). Monolayers of Vero cells in microtiter wells were infected with the reporter viruses plus compounds and assayed for β-galactosidase activity as described in Materials and methods.

Table 3
Possible mechanisms of inhibition as deduced by effects on β-galactosidase expression of the recombinant viruses

Inhibitor	Result	Possible mechanisms of inhibition
Aphidicolin	No effect on IE or E gene expression at concentrations which strongly reduce DE and L reports; stronger effect on L vs DE report	DNA synthesis inhibitor
XF884	Inhibited all reports at 5 and 15 μg/ml	Inhibition of viral entry and/or IE gene expression
BT318	Inhibited all reports modestly (30–50%) at a concentration (15 μg/ml) which inhibits the AE report by 80%	Inhibition of viral entry and/or IE gene expression
DS810	No effect on IE or E reports at concentrations which strongly reduce DE and L reports; stronger effect on L vs DE report	Possibly a DNA synthesis inhibitor, similar profile to aphidicolin
S5193	At concentrations which fully inhibit the AE report (10 μg/ml) the DE report was only reduced by 50% with only a very small effect (< 20%) on the L report	Different profile from aphidicolin, consistent with inhibition of viral assembly and/or egress
ER622	No effect on IE or E gene expression at concentrations which reduce the DE and L reports by 30 and 60%, respectively	Possibly weak DNA synthesis inhibition and/or inhibits assembly and/or egress

3.3. Discrimination between inhibition of entry / uncoating and IE synthesis

Inhibition of entry and/or uncoating or inhibition of immediate–early protein synthesis would give the same effect – a reduction in the IE report. Presumably, the effect would be transmitted throughout the viral cycle and would be recorded as a general reduction of all the other reports. This would be similar to what would be observed if a lower m.o.i. challenge had been used. Since the IE reports from both BT318 and XF884 were inhibited, experiments were performed to discriminate between these possibilities, particularly since XF884 showed variability in its ability to inhibit depending on when it was added postinfection.

To probe this, we asked whether there was a time dependence to the inhibitory effects of these compounds. Thus, Vero cells were inoculated with ICP4*lac*ZB at m.o.i. 5 and

XF884, BT318 and S5193 (negative control) were added to the cells as a function of time postinfection (0, 30, 60, 90, 105 and 120 min), versus no-compound control. Percent maximal β-galactosidase activity is shown Fig. 4A–C for assays performed 5 h p.i.

At 10 μg/ml, XF884 inhibited activity by > 95% when added before 30 min. At 60 and 90 min, inhibition was 74 and 40%, respectively. Beyond 105 min, XF884 was ineffective. The same time-dependent result was obtained at the lower XF884 concentrations, but the curves were shifted to lower inhibition. The results for BT318 differed. The activity of BT318 was dependent on the concentration (IC_{50} 11.4 μg/ml), but independent of the time of addition. As expected, S5193 did not inhibit the production of β-galactosidase at any concentration for any time point, consistent with the results from the panel of recombinants which had indicated that S5193 inhibited late in the viral life cycle.

4. Discussion

The identification of compounds capable of inhibiting herpes simplex growth is an important scientific and medical task. However, one of the difficulties in this kind of work is an understanding of where or how such compounds may be acting. In this work we have taken initial steps to simplify this problem by investigating the use of a panel of recombinant reporter viruses which signal where in the viral replication cycle inhibition may be taking place. The concept behind this approach is that different mechanisms of inhibition will affect each of the reporter viruses differently and that a profile of these effects can be used to assign possible modes of inhibition to newly discovered antiherpes agents. In a general way, one is asking whether the differential effects of antiviral agents can be dissected by a family of mechanism-specific assays, i.e., can one tell what 'class' of inhibitor a particular antiviral agent belongs to by noting its effects on each stage of gene expression driven by the viral program? An assumption in this approach is that the promoter-specific expression of β-galactosidase from a given recombinant virus can be considered to be a surrogate for the level of expression of the set of genes characteristic of the viral class of which that virus is a representative.

In an accompanying paper (Dicker et al., 1995) we report the construction of the HSV recombinant viruses used in this study. In particular, the ICP4*lac*ZB virus was shown to be a useful tool for characterizing the mechanisms of action of several antiherpes agents (a gD monoclonal antibody, IFN-γ and E3925, an experimental antiviral which inhibits viral entry). The viruses were also shown to quantitatively reproduce the results for IC_{50} determinations from standard plaque assays within 24 h in a microtiter plate format. Here we report the use of all 4 reporter viruses in a format for the rapid determination of where in the viral life cycle antiherpes inhibitors may be acting. When used in this fashion, the combination of viruses is referred to as the 'panel'. It should be noted that these recombinant viruses are TK-negative as a consequence of their method of construction. Thus, these viruses are less sensitive to inhibition by nucleoside-type antiherpes compounds (e.g., acyclovir) and may not be useful for the analysis of these types of agents. However, such agents can readily be

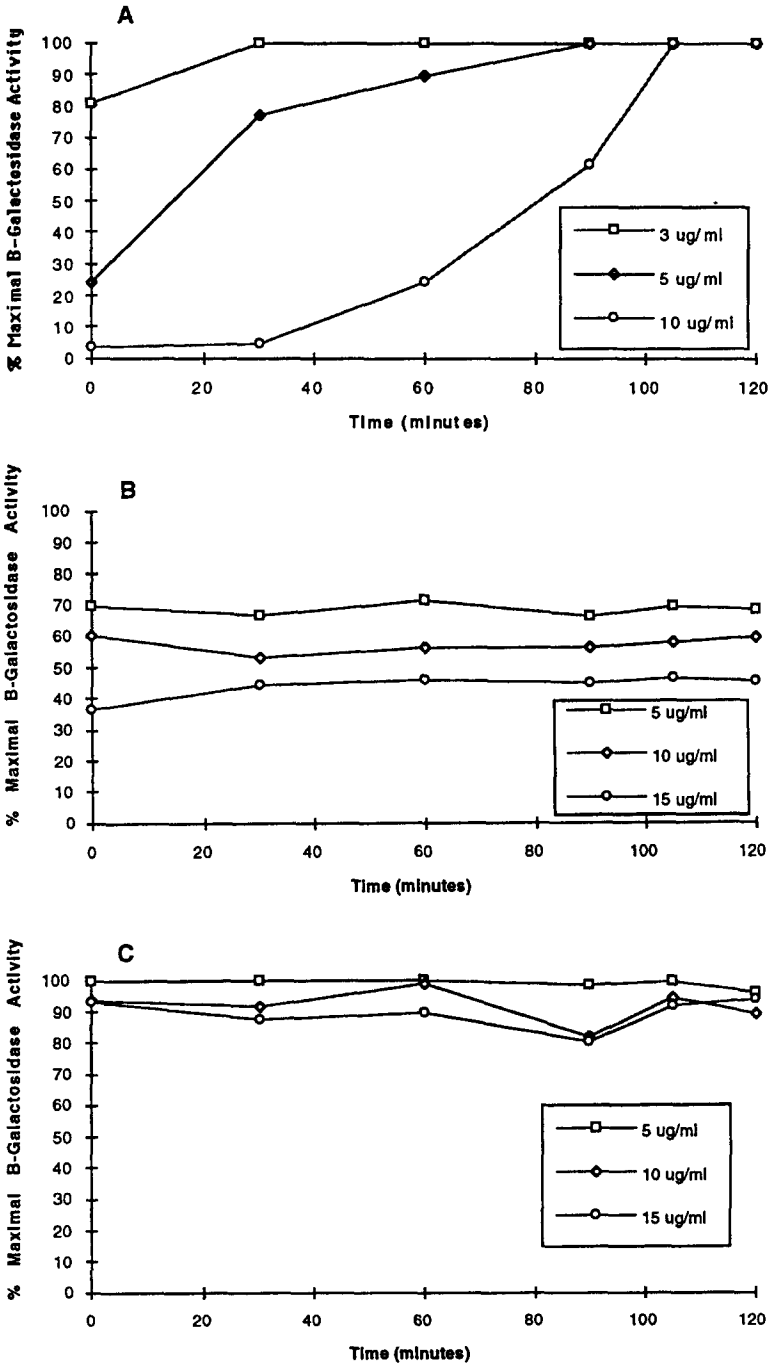

Fig. 4. Duplicate monolayers of Vero cells in microtiter wells were inoculated with 25 μl ICP4*lac*ZB virus at m.o.i. 5. XF884 (A), BT318 (B) and S5193 (C) (as a negative control) were added to the cells in 25 μl medium at twice the final concentration (including medium-only controls) as a function of time postinfection (0, 30, 60, 90, 105 and 120 min). β-Galactosidase activity was measured at 5 h p.i. and percent activity determined by comparison to the appropriate medium-only control.

identified by chemical structure and screened by standard plaque assays. The approach outlined here is intended to compliment normal screening procedures for the majority of agents for which the mechanism of antiviral activity is obscure.

To test the panel of viruses, we employed the known DNA synthesis inhibitor aphidicolin (Spadari et al., 1985) Expression from the immediate–early and early recombinant viruses (ICP4 and TK) was entirely unaffected whereas expression from the delayed early (gD*lac*ZA) and late (gC*lac*Z) viruses was reduced in a concentration-dependent fashion. There was a clear correspondence between the DE and L reports and the ultimate effect on viral output, as measured by the AE report. At low m.o.i. (0.05), where the growth of the virus must be evaluated after reinfection in the second cycle at 24 h p.i. (the signal was extremely low after 5 h with this m.o.i.), aphidicolin inhibited 50% of viral growth at 0.2 μg/ml and 85% of viral growth at 1 μg/ml. In contrast, aphidicolin had no effect on the expression of β-galactosidase, within the first cycle, from the ICP4 and TK viruses (up to a concentration of 3 μg/ml). One can conclude that both immediate–early and early gene expression was unaffected by this agent. However, there was a 60% loss of activity from the gD*lac*ZA virus at 1 μg/ml and an 80% loss of activity at 2 μg/ml. The results for the gC*lac*Z virus were more marked. At 1 μg/ml, the virus was inhibited by 85% while at 2 μg/ml the virus was inhibited by 96%. Thus, inhibition was confined to the panel members monitoring late, post-DNA synthesis onset expression. Also noteworthy is the intermediate effect on the DE report. This is consistent with the non-DNA synthesis-dependent component of gD expression (Johnson and Spear, 1984). Thus, the panel recapitulated the profile expected for a DNA synthesis inhibitor. This validation of our approach prompted us to evaluate antiherpes compounds of unknown mechanism of action.

The analysis of the compounds identified by plaque screening indicated that they inhibited HSV-1 at different points in the viral cycle. Controls indicated that the compounds were not toxic to the cells at the concentrations required to inhibit β-galactosidase activity or plaque growth by 50% and they were not direct inhibitors of β-galactosidase. Broadly, the compounds could be subdivided into two classes, those which inhibited the IE plus all other reports (XF884 and BT318) and those that only inhibited the DE and L reports (DS810, S5193 and ER622). XF884 and BT318 were further distinguished on the basis of very different effects on the inhibition of the IE report as a function of the time of their addition to cells inoculated with virus. The fact that inhibition by XF884 was a function of the number of minutes in which it was physically in contact with virus indicates that this compound acts prior to entry/uncoating of virus into cells. The underlying mechanism of this inhibition remains to be clarified, but an irreversible virucidal activity is a possibility. In contrast, BT318 showed no such time dependence, leading to the conclusion that it was acting after viral entry, but before or at the level of IE expression. Further experimentation on this compound can now focus on the molecular events within this window. DS810 appears to have nearly the same profile as that of aphidicolin, suggesting that it is an inhibitor of DNA synthesis. An assignment of potential mechanism of action of S5193 and ER622 is not so easily made. Clearly, these agents do not inhibit entry, IE or E events, but their profiles for inhibition of the DE and L reports do not exactly match that of aphidicolin. This suggests these compounds inhibit by additional or alternative mechanisms which

ultimately impinge on viral assembly and/or egress. The concentrations at which these compounds showed toxicity toward the Vero cells used in the study (Table 2) varied, but were higher than the concentrations required to inhibit by 50% in either the plaque or *lac*Z assays. This suggests these compounds were not exhibiting general cellular toxicity, though a more detailed analysis of effects on the cells would be required to completely rule this out.

The results described in this report illustrate the power of this kind of analysis to probe the mechanism of action of inhibitors of HSV growth. It is possible to adapt this methodology for use in large-scale screening efforts, with the goal of identifying compounds which inhibit at specific points within the herpes life cycle. The semi-quantitative nature of the report may also be used to investigate the additive or possibly synergistic effects of antiherpes agents which inhibit by different mechanisms, for example, an entry or IE inhibitor in conjunction with a DE/L inhibitor. Finally, the use of temporally regulated reporter genes may be useful in elucidating the mechanisms of action of inhibitors of other organisms, such as other viruses, bacteria and yeast and other systems, such as engineered mammalian cells.

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